Uptake and modification of coumarins by plants – a coherent phenomenon extending the concept of Horizontal Natural Product Transfer

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For My Mother, and The Soul of My Father

For My Family

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Abstract

It was recently stated that various alkaloids which are leached from rotting plants or exuded from living plants into the soil, are taken up by plants growing in the vicinity, designated as acceptor plants. The related phenomenon was denoted as "*Horizontal Natural Product Transfer*". It seemed reasonable that in analogy to alkaloids also other natural products should be taken up from the soil. Consequently, in this thesis, the uptake of phenolic natural products, and especially of coumarins was examined. Umbelliferone was chosen as a model compound since its uptake and derivatization can be easily detected and determined, due to the high fluorescence. Moreover, in contrast to other phenolic compounds, this coumarin is far more stable against oxidation.

Seedlings of various plant species. i.e., barley (*Hordeum vulgare* L.), radish (*Raphanus sativus* L.), pea (*Pisum sativum* L.), flax (*Linum usitatissimum* L.), and garden cress (*Lepidium sativum* L.) were grown in hydroponic media containing the coumarins. All plants took up umbelliferone by their roots and translocated it via xylem into the leaves, where it is accumulated to a high extent. In barley and garden cress, the imported umbelliferone was modified to yield scopoletin and esculin, respectively. Further analyses revealed that in both plant species, the imported umbelliferone is firstly hydroxylated to esculetin, which subsequently in garden cress is glucosylated to esculin or in barley is methylated to scopoletin. Based on the occurrence of non-derivatized umbelliferone in guttation droplets, it was assumed that the related modifications most probably take place in the leaves of the acceptor plants. In order to verify this assumption, isolated leaves and roots of barley were incubated with umbelliferone. Indeed, in the roots no derivatives were detected, and thus any derivatization in the roots could be ruled out. Surprisingly, in the cut leaves, in addition to scopoletin, large amounts of glucosidic derivatives, i.e., esculin and scopolin were detected. Obviously, the huge amounts of umbelliferone accumulated within the cut leaves induced or activated corresponding glucosyltransferases.

In order to further characterize the hydroxylation of the imported umbelliferone to esculetin, further investigations have been conducted. Since related modifications of xenobiotics are known to be catalyzed by cytochrome P450 enzymes, umbelliferone was applied to the seedlings together with naproxen, a well-known inhibitor of P450 enzymes. As expected, the conversion of umbelliferone to scopoletin in barley as well as the modification to esculin in garden cress was strongly reduced by the addition of naproxen. The results on the uptake of coumarins display that the horizontal natural product transfer represents a more general phenomenon in plant ecology, and quite different natural compounds are taken up by acceptor plants. In analogy to xenobiotics, in some plants, the imported substances are modified. However, these modifications are restricted to some species and are different in the various plant species. In consequence, these findings contradict the so-called "*Green liver concept*" that postulated a general detoxification process in plants.

Kurzzusammenfassung

Erst kürzlich wurde festgestellt, dass Alkaloide, die aus verrottenden Pflanzen ausgewaschen oder von lebenden Pflanzen in den Boden ausgeschieden werden, von anderen Pflanzen (Akzeptorpflanzen) aufgenommen werden. Dieses Phänomen wird als "horizontaler Naturstofftransfer" bezeichnet. Es erschien naheliegend anzunehmen, dass - analog zu Alkaloiden - auch andere Naturstoffe aus dem Boden aufgenommen werden. Daher wurde in dieser Arbeit untersucht, ob und in welchem Umfang phenolischen Naturstoffe, insbesondere Cumarine, von Akzeptorpflanzen importiert werden. Als Modellverbindung wurde Umbelliferon ausgewählt, da seine Aufnahme und Derivatisierung aufgrund der hohen Fluoreszenz leicht nachgewiesen und bestimmt werden kann. Darüber hinaus ist dieses Cumarin im Gegensatz zu vielen anderen phenolischen Verbindungen relativ oxidationsbeständig.

Für die Aufnahmestudien wurden Sämlinge verschiedener Pflanzenarten, wie Gerste (Hordeum vulgare L.), Rettich (Raphanus sativus L.), Erbse (Pisum sativum L.), Flachs (Linum usitatissimum L.) und Gartenkresse (Lepidium sativum L.) in hydroponischen Ansätzen kultiviert. Dann wurde Umbelliferon dem Medium hinzugefügt. Alle Pflanzen nahmen das Cumarin über ihre Wurzeln auf und verlagerten es über das Xylem in die Blätter, wo es sich in hohem Maße akkumuliert wurde. Überdies wurde in Gerste und Gartenkresse das importierte Umbelliferon zu Scopoletin und Esculin umgewandelt. Analysen ergaben, dass bei beiden Pflanzenarten das Umbelliferon zunächst zu Esculetin hydroxyliert wird, bevor es in Gartenkresse zu Esculin glucosyliert bzw. in Gerste zu Scopoletin methyliert wird. Aufgrund des Auftretens von Umbelliferon in Guttationstropfen und der Abwesenheit entsprechender Derivate wurde geschlussfolgert, dass die Modifizierungen des importierten Cumarins in den Blättern der Akzeptorpflanzen stattfinden. Um diese Annahme zu überprüfen, wurden isolierte Blätter und Wurzeln von Gerste-Sämlingen getrennt mit Umbelliferon inkubiert. Dabei zeigte sich, dass in den Wurzeln keine Derivate gebildet werden, und somit eine Derivatisierung in den Wurzeln ausgeschlossen werden kann. Überraschenderweise wurden in den abgeschnittenen Blättern zusätzlich zu Scopoletin große Mengen an Glucosidderivaten, also von Esculin und Scopolin, nachgewiesen. Offensichtlich haben die hohen Konzentrationen des in die inkublierten Blättern aufgenommenen Umbelliferons entsprechende Glucosyltransferasen induziert bzw. aktiviert.

Zur weiteren Charakterisierung der Hydroxylierung des importierten Umbelliferons, wurden zusätzliche Untersuchungen durchgeführt. Da bekannt ist, dass die analogen Umwandlungen von Xenobiotika durch Cytochrom P450-Enzyme katalysiert werden, wurde den Sämlingen zu¬sammen mit Umbelli¬feron auch Naproxen appliziert, einem bekannten Inhibitor von P450-Enzymen. Wie erwartet wurde durch Zugabe

von Naproxen in Gerste die Umwandlung von Umbelliferon zu Scopoletin sowie in Gartenkresse die Modifikation zu Esculin stark reduziert und somit nachgewiesen, dass Cytochrom P450-Enzyme an den Modifizierungen beteiligt sind.
Die Ergebnisse zur Aufnahme von Cumarinen zeigen, dass der horizontale Naturstofftransfer ein deutlich allgemeineres Phänomen darstellt als zunächst angenommen und ganz unterschiedliche Naturstoffe von Akzeptorpflanzen aufgenommen werden. In Analogie zu Xenobiotika können in einigen Pflanzen die importierten Substanzen modifiziert werden. Modifikationen kommen allerdings nur in Pflanzen einiger Arten vor. Zudem können sie sich in ihrer Qualität deutlich unterscheiden. Infolgedessen widersprechen diese Ergebnisse dem sogenannten „Green Liver Concept“, das einen allgemeinen Entgiftungsprozess in Pflanzen postuliert.

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Chapter 1: Introduction

Tremendous amounts of various anthropogenic chemicals are continuously discharged and introduced into the environment. Most of these compounds are taken up by plants - either by their roots, from which they are translocated into the leaves, or they are taken up directly by the leaves (Trapp, 2000; Boxall et al., 2006; Trapp and Legind, 2011). Since these substances are non-natural and "*strange*" to the plants that took them up, they are denoted as xenobiotics (Sandermann, 1992; Murray et al., 2006; Iovdijova and Bencko, 2010). In the past, most of the research on xenobiotics was focused on systemic herbicides, fungicides, and veterinary medicines. However, recently a further issue arose: the uptake of natural products from the soil. In the last decade, it became obvious that a massive number of herbal products such as spices, herbal teas, or phytopharmaceuticals, contain significant amounts of toxic alkaloids that are not inherently occurring in the related source plants. A comprehensive investigation of the European Food Safety Authority (EFSA) revealed that a tremendously high percentage of herbal products are contaminated by pyrrolizidine alkaloids (PAs) and nicotine (Mulder et al., 2015; European Commission, 2009). Indeed, in some cases, these contaminations are derived from the accidental co-harvesting of PA-containing weeds (Stegelmeier et al., 1999; Van Wyk et al.,2017). However, intensive studies unveiled that the occurrence of nicotine and PAs is - at least in part - due to their uptake from the soil (Selmar et al., 2015a; Nowak et al., 2016). This phenomenon was denoted as *Horizontal Natural Product Transfer* (Selmar et al., 2015a, b). In the case of nicotine, field studies showed that the alkaloids, which accumulated in acceptor plants, resulted from discarded cigarette butts (Selmar et al., 2015a, b). In contrast, the only PA containing litters present in the field, are decomposing weeds. However, even if there might be many of them growing nearby, only few individuals might be dead and rotting. Consequently, in addition to the leaching from rotted plant material, there must be another contamination

path for the PAs. In this context, it was demonstrated that PAs are also transferred from living donor plants to acceptor plants grown in the vicinity (Selmar et al., 2019). Accordingly, the phenomenon of *Horizontal Natural Product Transfer* had to be extended (Selmar et al., 2019).

Meanwhile, it was shown that many other alkaloids, like atropine, noscapine, harmaline, and vincamine are also taken up by plants (Yahyazadeh et al., 2017; Hijazin et al., 2020; Lewerenz et al., 2020). Moreover, various phenolic substances, which are known to exhibit ecological functions (e.g., as allelochemicals or as defensive compounds) are taken up by plant roots as well (Williams and Hoagland, 1982; Witzell and Martin, 2008). In addition, coumarins, which are secreted as chelating agents to facilitate the uptake of iron (Rodriguez-Celma et al., 2013; Rajniak et al., 2018) are also taken up.

As it is known for many xenobiotics (Trapp, 2000; Trapp and Legind, 2011), also the natural products are taken up from the soil by simple diffusion across the plasmalemma of the root cells, and no carriers are involved (Yahyazadeh et al., 2017). The only preconditions for this uptake are the physicochemical properties of the compounds (Nowak and Selmar, 2016; Yahyazadeh et al., 2017), i.e., their solubility in aqueous as well as in organic solvents[1].

Astonishingly, in many cases, the concentration of imported alkaloids in the acceptor plants ambiguously declined by the time (Selmar et al., 2015a; Nowak et al., 2016). Obviously, the substances are either degraded or modified. With respect to xenobiotics, it is well known that these substances are derivatized within the acceptor plants, e.g., by oxidation, hydroxylation, and conjugation (Sandermann,

[1] An appropriate assessment of this feature is given by the so-called log*P* value, the decadal logarithm of the distribution coefficient of a substance for an octanol and water system. All substances revealing a log*P* value between - 1 and 3 are able to passively cross the biomembranes (Trapp, 2000; Trapp and Legind, 2011; Limmer and Burken, 2014).

1994; Schaffner et al., 2002; Burken, 2003). According to the so-called "*Green liver concept*", these reactions are discussed to be part of a deliberate detoxification of xenobiotics (Sandermann, 1994; Burken, 2003). Thus, it is very likely that also the PAs taken up are bio-transformed in an analogous manner. Unfortunately, all attempts to determine putative derivatives of PAs in acceptor plants failed (Lewerenz, 2018). The main problem of these endeavors is due to the fact that the PAs - and also their putative derivatives - do neither reveal a significant UV absorbance nor a sufficient fluorescence and thus, they elude their detection by standard methods (Selmar et al., 2018).

Indeed, a classical approach for capturing putative derivatives would be the application of isotope-labeled substances. However, due to the related safety issues, alternatives are required. In this context, the employment of coumarins seemed to be very advantageous, since these substances and most of their derivatives can easily be detected because of their strong fluorescence (Jones and Rahman, 1994; Cao et al., 2019). However, since phenolic compounds exhibiting several hydroxyl groups are oxidized immediately, I chose the relatively stable umbelliferone (Figure 1-1) as an appropriate model substance in order to study its uptake and modification within different acceptor plants.

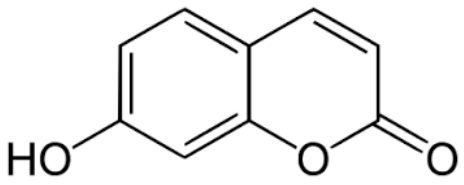

Figure 1-1: Structure of umbelliferone

Consequently, at first, it had to be studied whether or not umbelliferone is taken up by the roots of various acceptor plants. For this, an appropriate hydroponic culture system needed to be established. Subsequently, putative derivatives of the imported umbelliferone had to be detected, isolated, and identified. In addition,

certain clues regarding the enzymes involved in the modifications of umbelliferone should be gathered. Thus, the major aims and goals of this thesis are:

- Establishing a hydroponic system for the application of umbelliferone to various plants.
- Verifying the uptake of umbelliferone by various plant species.
- Detection, isolation, and identification of putative modification products.
- Elucidation of the modification site, i.e., roots or shoots.
- Providing information on the enzymes involved in the modification processes.

To achieve these goals, several different lines of experimental approaches have been followed:

1 - Designing and optimizing a hydroponic system that ensures a direct contact of the roots with the umbelliferone-containing medium, but preventing a direct transfer from the medium to the shoots. In this context, various sponges and plastic foams had been tested.

2 - HPLC analyses of plant extracts in order to verify the uptake of umbelliferone.

3 - HPLC analyses of plant extracts to detect putative derivatives of umbelliferone.

4 - Isolation and structure elucidation of the putative umbelliferone derivatives.

5 - Employment of enzyme inhibitors to gather relevant information on the enzymes involved.

6 - Application of coumarins to isolated organs (leaves, roots) to unveil the site of modification.

7 - Collecting and analyzing guttation droplets to elucidate the mode of translocation of umbelliferone and its derivatives with the plant seedlings.

Chapter 2: Scientific background

This study intends to further investigate the phenomenon of horizontal natural product transfer by analyzing the uptake and modification of coumarins in a hydroponic model system employing seedlings of various plant species.

To facilitate and expand the understanding of this recently discovered phenomenon, an actual overview of the scientific literature is presented in this chapter. In this context, the various aspects of the horizontal natural product transfer as well as those of the chemistry, biological activities, and the biosynthetic pathway of coumarins are outlined.

2.1 Horizontal natural product transfer

Plants are continuously exposed to different anthropogenic compounds, e.g., herbicides, or natural substances. Frequently, these substances designated as xenobiotics, are taken up by the roots of plants and are translocated into their shoots. In addition, allelopathic compounds that are synthesized and released into the soil to repress other plant species are also taken up by plants growing in the vicinity (Willis,1985; Inderjit and Duke, 2003).

Since decades, most of the research interest on the uptake was focused on human-generated compounds, e.g., herbicides and other xenobiotics. However, recently, the attention also turned to natural products, as being responsible for the contamination of many plant-derived commodities with nicotine (EFSA Reasoned Opinion, 2011), or pyrrolizidine alkaloids (EFSA Panel on Contaminations in the Food Chain, 2011; Mulder et al., 2015). Consequently, several related studies had been performed. This research unveiled that nicotine, leached out from discarded cigarette butts are taken up by the plants growing in the field (Selmar et al., 2015a), and, in the same manner, pyrrolizidine alkaloids (PAs) that had been leached out

from rotting PA containing weeds like *Senecio jacobaea*, are also taken up by plants grown nearby (Nowak et al., 2016).

Based on these findings the concept or the phenomenon "*Horizontal Natural Product Transfer*" was enunciated by Selmar et al., 2015a, b. This term denotes that the natural products which leached out from rotting plant material (donor plants) into the soil are taken up by the roots of other plants (acceptor plants) grown in the vicinity and then are translocated to the leaves (Selmar et al., 2015a, b).

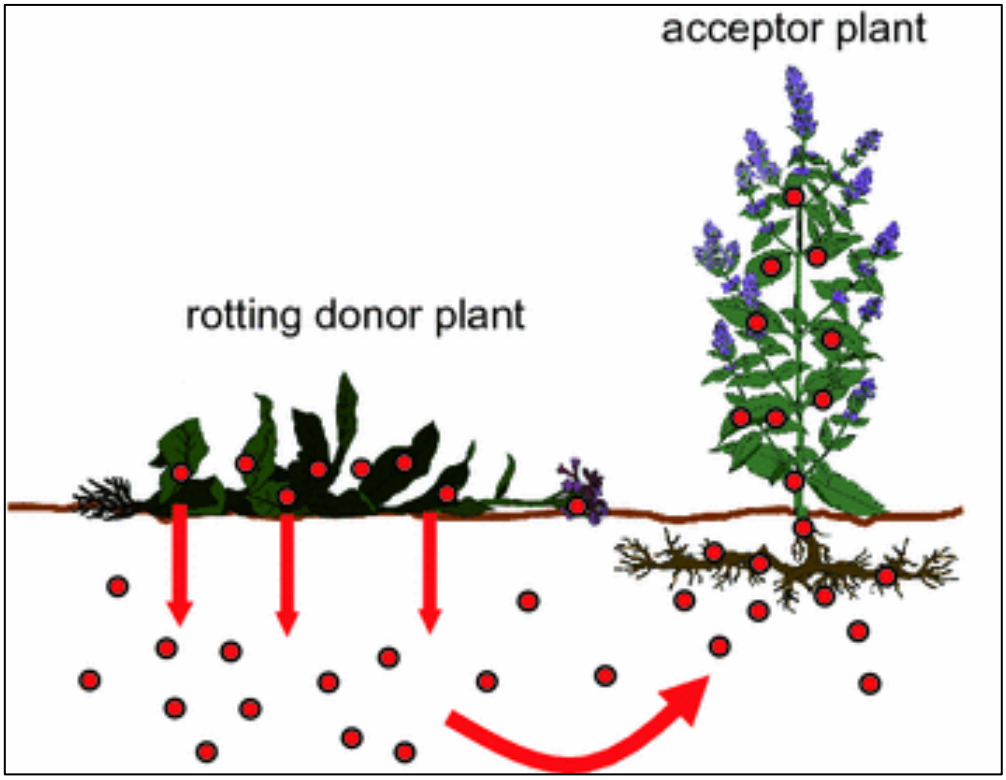

Figure 2-1: Originary concept of Horizontal Natural Product Transfer (Selmar et al., 2015b).

2.2 Broadening the concept of Horizontal natural product transfer: Transfer between neighboring living plants

It is well known that allelochemicals are exuded from plant roots or leaves into the soil, where they exert their allelopathic effects on the nearby plants (Nakano et al., 2003; Kalinova et al., 2007). With respect to the coherences mentioned above, the question arose, whether or not classical natural products might also be released

from the living plants into the soil, and if they are taken up analogously into acceptor plants. This presumption was studied by classical co-culture experiments.

As potential donors, PA-containing *Senecio jacobaea* plants had been grown in the same pots with non-PA-containing herbs, e.g., parsley (Nowak et al., 2017). After two months of co-culture, the parsley plants were harvested, extracted and their PAs content was quantified. A high amount of PAs presents in the parsley exhibited that the PAs synthesized in *S. jacobaea* had been transferred into the neighboring parsley acceptor plants. In principle, several options for such transfer are possible: a direct release of PAs into the soil from living plants due to an exudation (Walker et al., 2003), or an indirect transfer based on the leaching from dead or injured tissues, such as shed leaves or injured roots. Moreover, a straight transfer between the roots of the acceptor and donor plants because a close interaction of the roots might occur. Such root-to-root transfer requires some kind of natural root grafting (Basnet et al., 1993) based on a very tight contact of the neighboring roots (Selmar et al., 2019). Accordingly, the co-culture experiment was repeated under field conditions, where the herbal plants were grown at variable distances from the genuine PA-containing plants, to avoid the possibility of a close and direct contact between their roots (Selmar et al., 2019). After harvesting and analyzing the plant samples, the PAs were detected in all the acceptor plants grown at different distances. Thus, the possibility of root grafting could be excluded.

The most likely explanation for such transfer is based on the leaching of the relevant substances from dead or injured tissues. In this context, especially shed and rotting leaves as well as roots which had been injured in the course of their growth and penetration through harsh and stony soils.

The mulching experiments, in which dried PA-containing plant material was applied to various acceptor plants, revealed that all the genuine PAs present in the

donor plants were also found in the acceptor plants (Nowak et al., 2016). Surprisingly, in the co-culture experiment, the PAs spectrum of various acceptor plants was quite different from that of the donor plants, and many of the genuine PAs were not present in the acceptor plants. In most cases, just jacobine and its *N*-oxide could be detected (Selmar et al., 2019). Accordingly, these findings ruled out the possibility of leaching from shed leaves or bleeding of PAs from injured plant organs, respectively, since in these cases, the PA spectrum would be the same as in the mulching experiments. Thus, the PAs found in the co-cultured acceptor plants must have been exuded from the living plants, either from the leaves or from the roots.

By referring to the literature, only few hints are available that point to root exudation of alkaloids, i.e., that these compounds are selectively exported from the plant roots into the surrounding environment. Unfortunately, an unequivocal proof that a certain compound is exuded into the soil, is quite difficult, e.g., because of the frequently occurring root damages (Oburger and Jones, 2018). Accordingly, in most of the studies dealing with alkaloid exudation, hydroponic systems and organ cultures had been employed. In this manner, Toppel et al., (1987) studied the PA composition in the root cultures of *Senecio vernalis*. Whereas a large variety of PAs was present in the roots, only senkirkine was found in the culture medium. Therefore, the authors postulated that senkirkine was actively exuded. Analogously, the exudation of the indole alkaloid ajmalicine was outlined for hairy root cultures of *Catharanthus roseus* (Ruiz-May et al., 2009). Also, harmine and harmaline are reported to be exuded from transformed root cultures of *Oxalis tuberosa* (Bais et al., 2003), and nicotine from root culture of *Nicotiana tabacum* (Zhao et al., 2013). Based on these coherences, the concept of "*Horizontal Natural Product Transfer*" has to be expanded by including a transfer of natural products between living plants grown in proximity (Selmar et al., 2019), as shown in Figure 2-2.

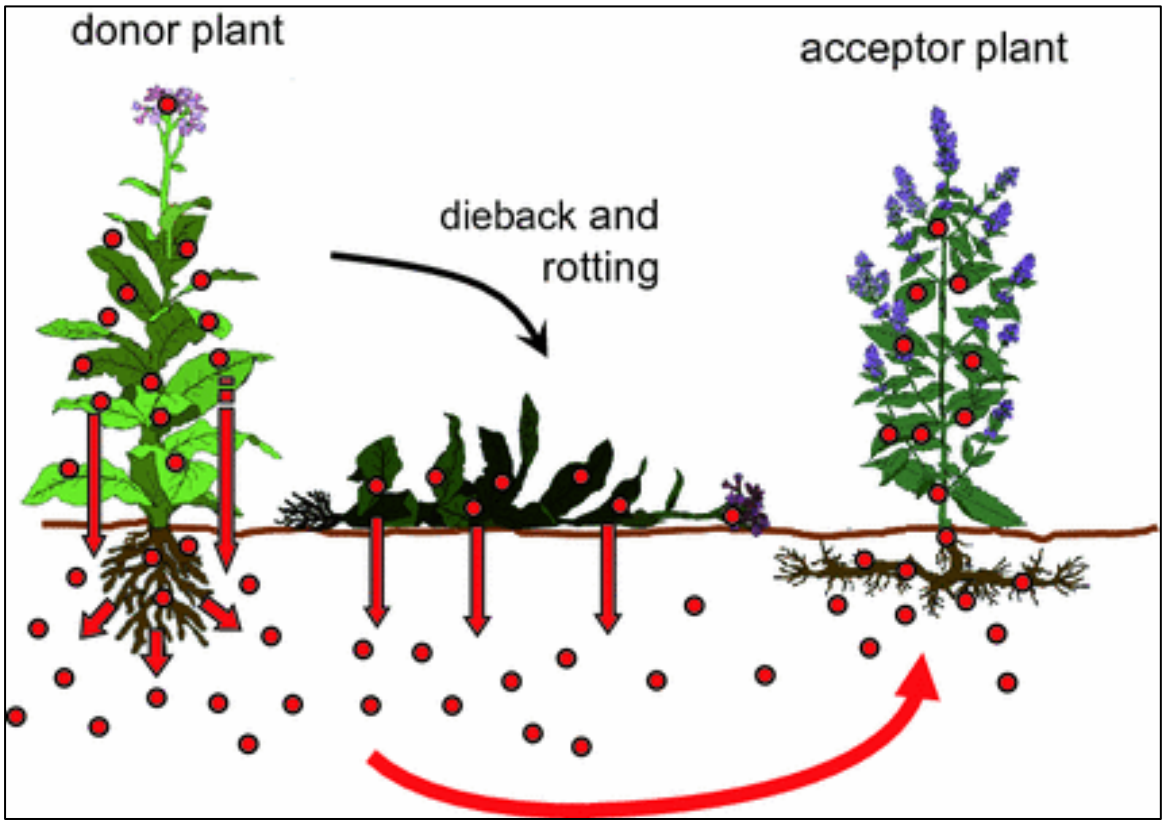

Figure 2-2: Broadening of the concept of "*Horizontal Natural Product Transfer*", the original concept was extended also to include the natural substances leached from living plants. Reprinted by permission from (Nowak et al., 2017). Copyright *2017* Springer International Publishing AG.

2.3 Uptake of natural Products – active or passive

It is well established that xenobiotics, in general, are taken up into the plants by simple diffusion and no transporters are required (Trapp and Legind, 2011). However, this diffusion depends on their membrane permeability and thus on their solubility in aqueous as well as in organic solutions. Accordingly, the ability of a certain compound to pass freely the biomembranes can roughly be estimated from *Kow*, representing its distribution coefficient between octanol and water. To date, mostly its decadal logarithm, i.e., the p*Kow*, frequently also denoted as log*P* is adopted (Cronin and Livingstone, 2004; Trapp, 2004). Based on the physicochemical requirements, it is supposed that substances revealing log*P* values between −1 and 3 generally diffuse passively through biomembranes

(Limmer and Burken, 2014). There is no reason why these coherences and rules elaborated for xenobiotics should not also apply for natural products.

In consequence, various natural products had been tested for their ability to be taken up by variable plant species. Yahyazadeh et al., (2017) reported that all alkaloids revealing log*P* values between -1 and 3 indeed are taken up, like tropane, purine, indole, and benzylisoquinoline alkaloids. On the contrary, the quaternary alkaloids, e.g., coptisine, palmatine, or berberine are not taken up since these alkaloids are exhibiting a permanent positive charge that hinders their diffusion across biomembranes, expressed by their negative log*P* values (Yahyazadeh et al., 2017).

Nonetheless, in addition to the log*P*, we have to take into consideration that other factors affect the uptake of alkaloids. In this context, the pH of the soil or the medium is the most relevant property: the pH strongly affects the protonation and thus their hydrophilic character. Due to the positive charge of protonated alkaloids, they cannot any more diffuse through the biomembranes (Trapp, 2009; Nowak and Selmar, 2016; Hijazin et al., 2020). A further factor is related to the overall solubility of alkaloids (Hijazin et al., 2020). In acidic media, due to their positive charge, the protonated alkaloids are much better soluble than in neutral or alkaline media, respectively, where these natural products are present as free bases. In consequence, the p*ka* value of an alkaloid strongly impacts its solubility and thus the share of molecules that is able to diffuse across bio-membranes. Thus, both factors, log*P* and p*ka*, will determine the capability as well as the extent of an alkaloid to simply pass biomembranes (Hijazin et al., 2020).

In this context, it has to be mentioned that conjugated compounds, such as glucosides or other hydrophilic derivatives are not able to pass the biomembranes due to their enhanced hydrophilicity, which of course, is expressed by their corresponding negative log*P* values. These coherences are nicely displayed by the

differences in membrane permeability of coumarins. Whereas the basic coumarins, e.g., esculetin or scopoletin, simply diffuse across biomembranes, their corresponding glucosides, i.e., esculin and scopolin, are membrane impermeable. As outlined below, the enzymatic interconversion of the different forms strongly influences the membrane permeability and thus their cellular localization.

As outlined above, several factors affect the membrane permeability of a certain natural product and thus its putative uptake from the soil into acceptor plants, i.e., the pH of the soil and the physicochemical properties of the compound. However, a further point has to be taken into consideration, i.e., the degradation of the natural compounds by the microorganisms colonized in the soil. Accordingly, the actual amount of a certain compound present in the soil is also massively affected by the soil microbiome, which in turn affects the extent of its uptake by acceptor plants (Fetzner, 1998).

2.4 Uptake of other natural products

Alike nicotine, PAs, and various further alkaloids, also other natural products could be taken up by the plants, provided that their physicochemical properties are compatible with the requirements of passive diffusion through biomembranes. In this context, phenolic compounds are suitable candidates. Various phenolics are known to be either leached out from rotted plant materials or are exuded from living cells and reveal certain ecological functions, e.g., as defensive compounds against pathogens, allelochemicals affecting the crop yield (Witzell and Martin, 2008; Li et al., 2010). Thus, these substances should be taken up by acceptor plants. Moreover, various coumarins are known to be exuded from the roots to facilitate the uptake of iron, especially in the alkaline soil (Rodriguez-Celma et al., 2013; Rajniak et al., 2018). Whereas for the uptake of corresponding positively charged iron-coumarin complexes a transporter is required, the genuine coumarin is able to freely diffuse through biomembranes.

Indeed, in the past, several experiments had been performed, which display the ability of coumarins to pass passively the biomembranes. Yet, these experiments aimed to analyze putative modifications of these compounds in plant cells. Werner and Matile (1985) reported that isolated protoplasts from the mesophyll of barley leaves take up exogenous scopoletin and esculetin and glucosylate these coumarins to their corresponding glucosides, i.e., scopolin, esculin, and cichoriin. Since the rate of uptake was proportional to their concentration in the media and the kinetics of their transport didn't show any saturation state, they concluded that these aglycones simply diffuse across the plasmalemma of the protoplasts. In contrast, the translocation of the glucosides (which are membrane-impermeable) into the vacuoles, requires an active transport. These results had been confirmed by several other researchers. In this sense, cell suspension cultures, e.g., *Lithospermum erythrorhizon*, *Gardenia jasminoides,* and *Nicotiana tabacum* take up esculetin from the culture medium and glucosylate it to esculin (Tabata et al., 1984). In another experimental series using cell cultures of *Datura, Lithospermum, Perilla,* and *Catharanthus*, it was shown that these cell cultures were able to take up various other phenolic compounds such as daphnetin or umbelliferone, and glucosylate them (Tabata et al., 1988). However, these findings on the uptake and modification of different substances had never been discussed for a putative uptake by the plant roots.

Further studies confirmed that many other compounds can pass biomembranes and quite different classes of organic compounds are taken up by acceptor plants. In this context, the allelochemicals benzoxazolinone (BOA) and biochanin A are known to be taken up by plants (Schulz and Wieland, 1999; Shajib et al., 2012). Furthermore, Hurtado et al., (2016) demonstrated that many so-called "emerging organic contaminants" such as pharmaceuticals and personal care products are taken up by lettuce plants.

2.5 Further broadening of the "Horizontal natural product transfer" concept.

Apart from the general phenomenon of an uptake into putative acceptor plants, two other issues have to be considered: a putative modification of the imported substances and the translocation within the acceptor plants. In this context, two major aspects have to be elucidated, i.e., do the putative modifications occur in the roots or the leaves, and is the translocation of the natural substance taken up (or its potential derivatives) performed via xylem or phloem.

2.5.1 Modification of the imported substances

The first hint for modifications of the imported natural products was noticed when quantifying the PAs taken up by a certain plant species. The standard HPLC methods revealed that the content of the PAs decreased by time (Selmar et al., 2015; Nowak et al., 2016). However, when altering the quantification method to the so-called sum parameter method, quite other results had been achieved. The sum parameter method is based on the quantification of the necine base[2]. Accordingly, in addition to all genuine PAs present in the donor plants, also putative derivatives of PAs still containing the PA-backbone, i.e., the necine base, will be determined (Cramer et al., 2013). The related studies displayed that the PA content detected by the sum parameter methods is much higher than the putative content when applying the standard method. In contrast to a decrease in the PA content detected by the standard HPLC, a continuous increase was recorded. These data unequivocally demonstrated that a large share of the imported PAs is modified (Selmar et al., 2018). Unfortunately, due to their weak UV absorbance and lack of appropriate fluorescence, the modified products could not be determined so far,

[2] This new approach is based on the de-esterification of PAs employing a $LiAlH_4$. Accordingly, from each PA containing the basic necine base, retronecine is released, which subsequently is derivatized and quantified employing HPLC coupled to an ESI–MS/MS device. Since the $LiAlH_4$ treatment also reduced all PA-*N*-oxides, this method determines all known PAs and their *N*-oxides as well as potential metabolites.

and no information on the modification mechanisms is currently available (Lewerenz, 2018). Nonetheless – PAs are modified.

Based on the finding that PAs are modified in the acceptor plants and the data on the glucosylation of coumarins taken up by cell suspension cultures, the question arose: are also the other compounds modified in the acceptor plants after their uptake. In this context, we have to consider that a wide array of xenobiotics is known to be modified in the acceptor plants - a phenomenon representing the basis for the "*Green Liver Concept*" (see below, 2.6).

In order to clarify the fate of natural products in acceptor plants, only few hints (see above) are available and there is a massive need for further research. Yet, in corresponding approaches not only the genuine substances taken up but also their putative derivatives have to be traced easily. Accordingly, this thesis is focused on this issue to further elucidate this actual topic by employing coumarins. These natural compounds are known to exhibit a strong innate fluorescence, which also is maintained in many of their putative derivatives. Applying coumarins as model compounds, solid information and the first proof of biotransformation of imported natural products could be elaborated (see "Results" chapter, section 4.2).

2.5.2 Translocation of PAs

After finding the source of herbal products contamination by PAs and nicotine, which were leached out to the soil then taken up by the plants (Selmar et al., 2015b; Nowak et al., 2016), the question arose how the alkaloids had been translocated from the roots into the shoots. Based on the analyses of guttation droplets, Nowak (2017) showed that the alkaloids are transported via the xylem, driven by transpiration. Accordingly, they accumulate in the leaves of the acceptor plant, and not in the typical physiological sinks, such as flowers, seeds, or fruits, which are supplied by the phloem (Selmar et al., 2015b; Nowak

et al., 2016). This is quite different than the situation of the PA-translocation in genuine PA-containing plants. It is well established that the endogenous PAs synthesized in the roots are translocated via phloem as PA-*N*-oxides into the flowers and seeds (Hartmann et al., 1989; Witte et al., 1990). In contrast to the free alkaloid bases, their *N*-oxides are very hydrophilic and are not able to pass biomembranes passively. Accordingly, the PA-*N*-oxides are retained in the phloem and translocated and deposited in the sink tissues, e.g., fruits and seeds. The free bases of PAs, when present in the phloem, would immediately diffuse into the xylem and be trapped according to the ion trap mechanism (Matile, 1976) driven by the different pH values in the xylem and phloem (Nowak and Selmar, 2016). In other words, the physicochemical properties determine the mode of translocation, i.e., via phloem into sink tissues (seeds, fruits) or via xylem into the leaves. Based on these coherences, any modification of natural products imported into acceptor plants distinctly impacts the translocation. Moreover, we have to be aware that putative modification might be performed already in the roots or, alternatively, might occur in the shoots. A nice example for the latter option was recently reported from Lewerenz et al., (2020) who showed that harmaline taken up by the roots of barley seedlings is translocated into the leaves, where it is oxidized to harmine. Furthermore, the stilbene resveratrol, which is taken up by barley seedlings is subsequently glucosylated (Selmar et al., 2018).

Due to the increasing knowledge on the modification of imported natural products, the basic concept of "*Horizontal Natural Product Transfer*" has to be further extended as shown in the figure below.

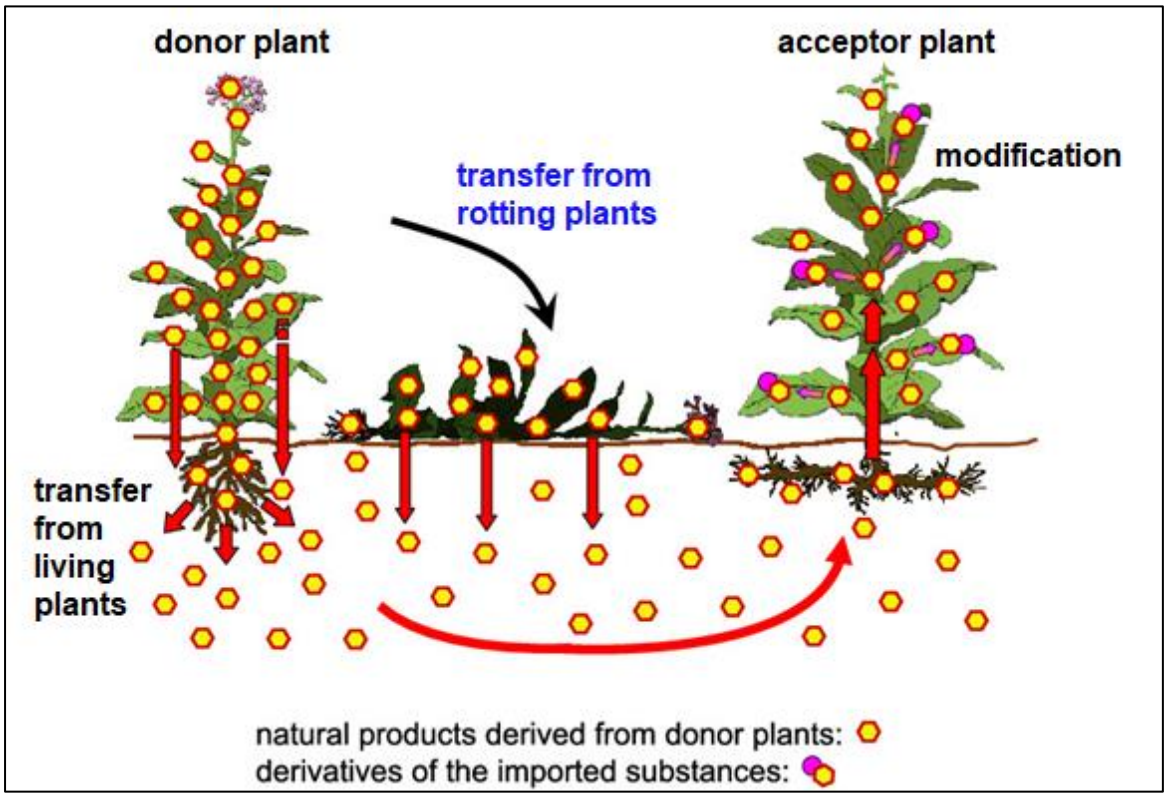

Figure 2-3: Modification of the natural compounds after their uptake in the acceptor plants. Adapted with permission from (Selmar et al., 2019). Copyright 2019 American Chemical Society.

2.6 Modifications known from the "*Green Liver Concept* "

As mentioned before, plants growing in soils polluted by xenobiotics, take up these substances (Boxall et al., 2006; Trapp and Legind, 2011). Detailed analyses revealed that in many cases these substances are modified into their corresponding derivatives, which most frequently are quite hydrophilic and thus far less membrane-permeable than the original xenobiotics taken up. Consequently, these derivatives are trapped in certain compartments. Based on these coherences, the "*Green Liver Concept*" was formulated. According to this theory of a deliberate detoxification, plants performed a series of reactions or biotransformation processes that change the properties of the imported substances and convert them into hydrophilic and non-toxic compounds. These reactions include hydroxylation, oxidation, or reduction (phase I) and conjugation (phase II). Finally,

in phase III, the derivatives are sequestered and deposited or excreted in certain compartments. i.e., vacuoles or apoplastic space (Sandermann, 1994; Burken, 2003). In case of a general implementation of this concept, there should be no mind that – in the same manner - also the natural substances taken up by the plants have to be modified analogously.

Indeed, with respect to any modification of natural products randomly taken up from tainted soils, up to now, no valid data are available. However, there are several studies dealing with the uptake and modification of allelochemicals, e.g., biochanin A or benzoxazolin-2(3H)-one (BOA). Intriguingly these compounds are metabolized or detoxified, respectively, in different plants differentially (Schulz and Wieland, 1999; Shajib et al., 2012). Moreover, it is well established that the inhibitory effect of juglone strongly differs between various plant species (e.g., Rietveld, 1982; Kocacë Aliskan and Terzi, 2001). Obviously, juglone – just like biochanin A or BOA – is modified differentially within the plants of different species. When considering these coherences, it could be concluded that a deliberate detoxification process, as postulated in the context of the green liver concept, might not be applicable – at least not in all cases. This, however, displays that the theory of a general detoxification system does not pertain.

Alternative to a "deliberate" detoxification process, it could be thought that the imported compounds are accidentally metabolized, e.g., by the numerous enzymes involved in secondary metabolism, which apparently vary in different plant species. In this context, the substrate specificity of the enzymes involved is of special concern. Indeed, in the past, it was postulated that the enzymes involved in secondary metabolism are highly specific (e.g., Hartmann, 1996; Wink, 1997). But, meanwhile, we have learned that the substrate specificity of enzymes is far lower than initially assumed (e.g., Atkins, 2015). The property that one enzyme is able to catalyze the conversion of several substrates, is nowadays denoted as

enzyme promiscuity (Khersonsky and Tawfik, 2010; Copley, 2014), in the strict sense, as one manifestation of enzyme promiscuity (Khersonsky and Tawfik, 2010; Copley, 2014; Kreis and Munkert, 2019). In consequence, the differences observed in modifying imported allelochemical or randomly taken up natural substances might be ascribed to different markedness of enzyme promiscuity as well as differences in the spectrum and content of enzymes. Accordingly, the principal question arises whether the modification of imported substances corresponds to a "deliberate detoxification process", as postulated by the "*Green Liver Concept*" or is due to an "accidental modification" due to enzyme promiscuity, and a broad substrate specificity of enzymes.

2.7 Coumarins

Secondary plant products or also denoted as phytochemicals are described to exhibit various functions in the complex interaction of plants with their environment, e.g., by protecting them against biotic and abiotic factors and stressor, e.g., drought, herbivore, and pathogenic attack (Gibson et al.,1998; Hasler et al., 1999; Mathai, 2000; Saxena et al., 2013). Apart from flavonoids and lignin, coumarins represent the most frequent and widely distributed phenolic natural products in nature (Venugopala et al., 2013). They originate from the phenylpropanoid pathway and represent derivatives of benzopyrones (1-benzopyran-2-ones; Figure 2-4).

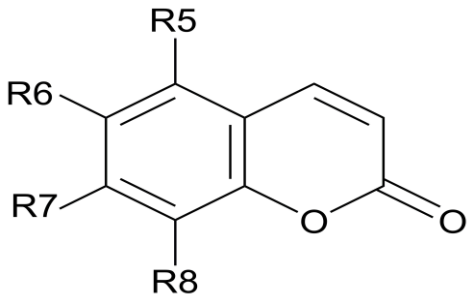

Figure 2-4: Basic chemical structure of coumarins

Indeed, for the comprehension of the research presented in this thesis, no detailed knowledge of the various aspects of biology, phytochemistry, and biochemistry of coumarins is required. However, with respect to various lines of argumentation and conclusions outlined in the discussion, basic information on coumarins is necessary. Accordingly, in the next sections, these distinct issues are presented.

2.7.1 Classification of coumarins

Coumarins are sub-classified according to their structures as simple coumarins (1,2-benzopyrone), 7-oxygenated coumarins (furanocoumarins), pyranocoumarins (benzodipyran-2-ones), and phenylcoumarins (benzo-benzopyrones; Murray et al., 1982; Murray, 1991; Este´vez Braun and Gonza´lez, 1997). Simple coumarins are the most common types in plants. The basic structure, i.e., coumarin, reveals a sweet, vanilla-like odor. Its hydroxylated and methoxylated derivatives (e.g., umbelliferone, scopoletin, or esculetin), and their glycosides, respectively, are known to represent important protective substances, either as phytoanticipins or as phytoalexins (Edwards et al., 1997; Wink, 2003; Shimizu et al., 2005).

2.7.2 Biosynthesis of coumarins in plants

Coumarin biosynthesis had been elucidated by employing radioactively labeled precursors (Brown, 1981). These analyses revealed that they are synthesized by the classical phenylpropanoid biosynthetic pathway via shikimic acid. Yet, in contrast to most other phenylpropanoids, where the aromatic moiety is hydroxylated in *para*-position (yielding in *p*-coumaric acid), the characteristic step in coumarin biosynthesis is an *ortho*-hydroxylation (Figure 2-5). In the resulting *o*-coumaric acid - due to the strong interaction of the OH-group and the carboxyl group - the *cis*-position of the *trans-cis* isomerization is favoured and an esterification occurred

that yields in the production of the lactone ring. Accordingly, *ortho*-hydroxylation represents the key step in coumarin biosynthesis.

Just in the same manner as it is known for other phenylpropanoids, also in the case of coumarins, a *p*-hydroxylation could take place. Moreover, in some cases, further hydroxyl groups may be introduced. However, the order of the related hydroxylation steps might vary. In some plants, *o*-hydroxylation occurs, when the *p*-hydroxy-group is already introduced, e.g., when *p*-coumaric acid is *o*-hydroxylated to yield umbelliferone (7-hydroxy coumarin). This reaction is catalyzed by a *p*-coumaric acid 2-hydroxylase. In various plants, umbelliferone (7-hydroxy coumarin) is considered to be the parent molecule for many simple coumarins (Brown, 1985), such as esculetin (6,7-dihydroxy coumarin), which – at least in *Daphne mezereum* - is methylated to yield scopoletin (7-hydroxy-6-methoxy coumarin). By contrast, in *Hydrangea macrophylla,* scopoletin is produced directly by an *o*-hydroxylation of ferulic acid, and no esculetin acts as an intermediate. In the same manner, also caffeic acid could be directly converted to esculetin without umbelliferone as an intermediate. These examples outline that a certain coumarin could be synthesized in various plants by different routes. Consequently, not only the putative precursors (cinnamic acid, *p*-coumaric acid, caffeic acid, and ferulic acid) may vary, but also the order of hydroxylation and methylation steps. Accordingly, there are various controversial opinions concerning the order of events. Furthermore, the situation becomes far more complex, since in several cases the various hydroxyl groups also could be glucosylated or esterified with shikimic, respectively. Without question, also the order of the related reactions is discussed controversially (Kindl, 1971; Brown, 1986).

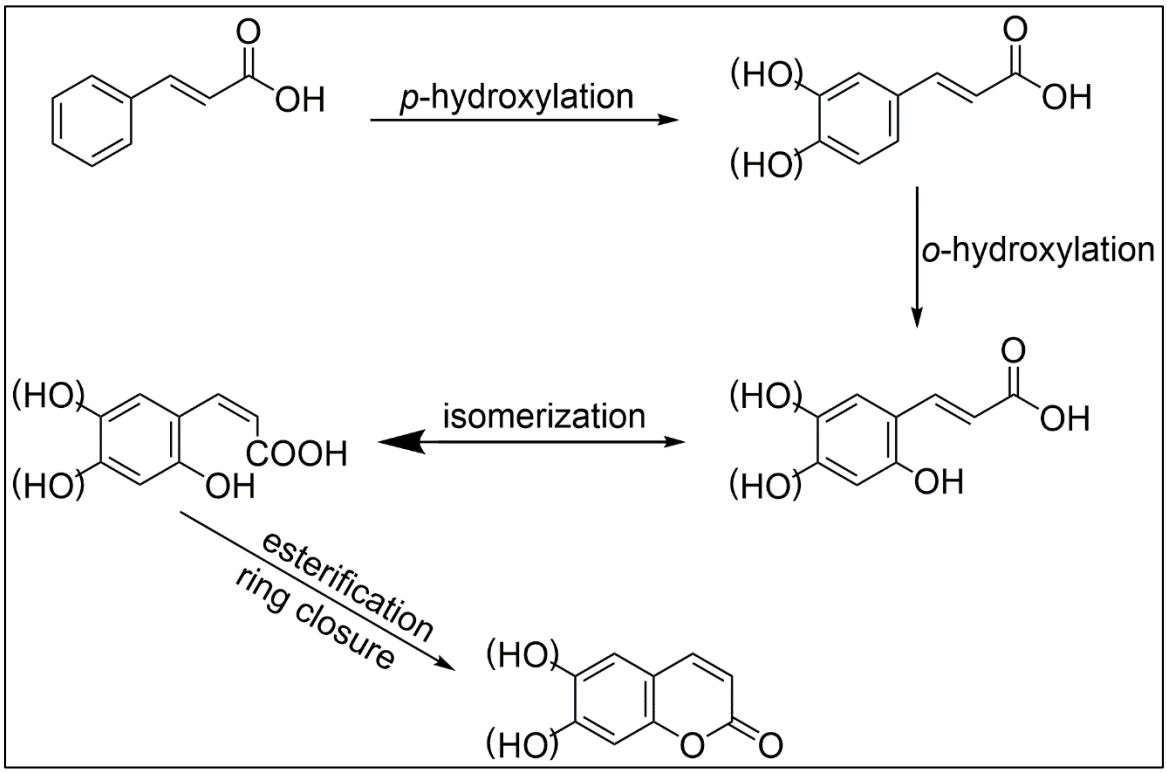

Figure 2-5: Generic steps in coumarins biosynthesis.

2.8 Coumarins and ecological biochemistry

Coumarins - like all other natural products - reveal numerous ecological activities, which are markedly determined by the various substitutions of their rings. In consequence, coumarins also exhibit quite different pharmacological activities (see below, section 2.9).

2.8.1 Protective agents

Coumarins are well known for their various effects on plant defense. As phytoanticipins, their presence protects the plants against herbivores and numerous pathogens (Zobel and Brown, 1995; Wink, 2003). In various cases, the accumulated compound itself exhibits the defense effect, e.g., in the wild parsnip (*Pastinaca sativa*) furanocoumarins are reported to protect the plants against the cabbage looper (*Trichoplusia ni*; Zangerl, 1990) and various other insect species (Berenbaum, 1978; Berenbaum and Feeny, 1981). In tobacco, the accumulated

scopoletin is involved in plant resistance to viral infection (Chong et al., 2002). By contrast, in *Melilotus alba*, the repelling coumarin is only produced as a response to herbivore attack: when the cells are disintegrated, the precursor, i.e., the glucosylated o-coumaric acid is hydrolyzed by the action of β-glucosidases, and the repelling coumarin is produced spontaneously (Poulton et al., 1980) and could exhibit its toxic effect on insects (Pavelo et al., 2019).

However, the most prominent insights demonstrating the protective role of coumarins are based on their relevance as phytoalexins. As one of the most intriguing examples, scopoletin is synthesized *de novo* when plants, such as potatoes, rubber trees, sunflowers, tobacco, and others, are attacked by pathogenic microorganisms (Gnonlonfin et al., 2013). Due to its fungitoxicity, scopoletin protects the host plant against invading pathogens. This was nicely represented by the accumulation of the bright blue fluorescing coumarin derivative in the tissues of potato infected with *Phytophthora infestans* (Hughes and Swain, 1960). Scopoletin is exuded from the living cell into the apoplast of the infected rubber tree leaves as a response to a fungal attack (Garcia et al., 1995; Silva et al., 2001; Lieberei, 2007). Following the infection, catalyzed by a UDP-glucosyl transferase, scopoletin is glucosylated to yield its glucoside, i.e., scopolin, and stored in the vacuoles. This glucosylation is considered as a mean of detoxification (Taguchi et al., 2000). As a result, when the plant cell is attacked again by a pathogen, in the course of decompartmentation as a result of cell rupture, the stored scopolin comes into contact with apoplastic β-glucosidases and is cleaved (Morant et al., 2008, Ahn et al., 2010). Per definition, the produced bioactive aglycone scopoletin represents a phytoanticipin, although the same molecule had previously been synthesized as typical phytoalexin.

2.8.2 Chelating properties and significance for the uptake of iron

Apart from their protective and defense roles, coumarins also have a certain relevance for the primary metabolism. Studies employing *Arabidopsis thaliana* revealed that coumarins play a significant role in the uptake of ferric ions (Fe^{3+}). In this context, the coumarins act as iron-chelating agents, which are exuded into the soil (Fourcroy et al., 2014; Schmid et al., 2014; Schmidt et al., 2014). This in particular is relevant, when the iron is poorly available, i.e., in neutral and alkaline soil, where the solubility of Fe^{3+} is quite low (Hindt and Guerinot, 2012). Because of the presence of two adjacent hydroxyl groups, especially the catecholic coumarins such as esculetin, fraxetin, and sideretin are efficient chelators, and thus most capable to be involved in the iron uptake (Schmid et al., 2014, Siso-Terraza et al., 2016; Rajniak et al., 2018). Whereas the exudation of coumarins into the soil - due to their membrane permeability - is passive, the re-import of the membrane impermeable Fe^{3+}-coumarin complexes requires a specific transport system (Curie et al., 2001; Murata et al., 2006).

2.9 Pharmacological activity

As outlined above, coumarins exhibit quite different pharmacological effects. They are well-known to exhibit a strong anticoagulant activity: because of their structural similarity to vitamin K, they act as a competitive inhibitor in the coagulation cascade pathway (Goodman and Gilman's, 2006; Venugopala et al., 2013).

Like many other phenolic products, coumarins also have the ability to scavenge and detoxify reactive oxygen species (ROS). Moreover, coumarins can inhibit xanthine oxidase, which generates ROS during xanthine biosynthesis (Lee et al., 2007; Bajerova et al., 2014).

Furthermore, many coumarins are potent anti-inflammatory drugs by preventing cell-adhesion molecules (Goodman and Gilman's, 2006; Witaicenis et al., 2013), and by stimulating phagocytosis and proteolytic enzymes that remove proteins and fluids from injured tissues, coumarins also aid in wound healing (Piller, 1975).

Additionally, several coumarins reveal antiviral effects, e.g., coumarin, which has been used to prevent recurrences of cold sores caused by HSV-1 in humans (Berkada, 1978). Also, various antibacterial activities have been reported (Stavri et al., 2003; Céspedes et al., 2006). However, as the effectivity of the natural coumarins is relatively low, various derivatives had been generated, e.g., Novobiocin or Clorobiocin, two amino derivatives, which exhibit a broad-spectrum activity towards Gram-positive bacteria, including methicillin-resistant strains of *staphylococci* species (Schio et al., 2000).

Chapter 3: Materials and methods

3.1 Plant material and chemicals

Seeds of *Hordeum vulgare* L., *Raphanus sativus* L., *Pisum sativum* L., *Linum usitatissimum* L., *Lepidium sativum* L. were purchased from commercial markets (Reformhaus Bacher; Samenhaus Knieke, Braunschweig, Germany).

Umbelliferone (≥ 98%) was purchased from Feinbiochemica. Skimmin (≥ 98%) was purchased from Cfm Oskar Tropitzsch GmbH. The following coumarin standards (≥ 98%): esculetin, esculin, scopoletin, and scopolin were supplied by Roth. Acetonitrile (HPLC grade) and Methanol (HPLC grade) were purchased from VWR. Acetic acid (HPLC grade) was supplied by Fisher Scientific. Naproxen (≥ 98%) was purchased from Sigma-Aldrich. N-D-Glucosyl piperidine and N-D-Galactosyl piperidine were prepared by Prof. Dr. Dirk Selmar according to (Hodge and Rist, 1952)

3.2 Hoagland Solution

Modified-Hoagland solution (Table 3-1) was prepared according to Johnson et al., (1957).

Table 3-1: Components of Hoagland's solution.

	Compound	**Concentration of stock solution (g/L)**	**Volume of stock solution**
Macronutrients	KNO_3	101.10	6.48
	$Ca(NO_3)_2.4H_2O$	236.2	2.77
	$(NH_4)\ H_2PO_4$	115.08	1.00
	$MgSO_4.7H_2O$	246.49	4.18
Iron supplement	$FeSO_4$	3.37	5
	Na-EDTA	2.08	
Micronutrients	H_3Bo_3	2.86	1.14
	$MnSO_4.5H_2O$	1.540	
	$CuSO_4.5H_2O$	0.080	
	$ZnSO_4.7H_2O$	0.880	
	$Na_2MoO_4.H_2O$	0.025	

All these macro- and micro-components were added together, then completed to one liter by distilled water, the pH is adjusted to 5.5-6 using Schott pH-Meter CG 841. This solution provides the nutrients necessary for supporting the growth of a large variety of plant species grown in a hydroponic system.

3.3 Optimizing a hydroponic system and seedlings preparation

A hydroponic system for the uptake of umbelliferone and other coumarins was established employing seedlings of barley (*Hordeum vulgare*), radish (*Raphanus sativus*), pea (*Pisum sativum*), flax (*Linum usitatissimum*), and garden cress (*Lepidium sativum*). Two methods were tested to develop a suitable hydroponic system. After several modifications and optimization steps for both of them, method 2 was found to be the most efficient and fitted one to perform this study. A comparison between these two methods will be outlined in the "Results" chapter (section 4.1).

In both methods, the seeds of all the employed plant species were germinated. Four days after germination, the hypocotyl of each single seedling was carefully wrapped by a small piece of sponge (5×25 mm).

Method 1: Each wrapped seedling was inserted in a test tube (18×160 mm) containing Hogland's medium. Every test tube holds a single seedling and is aerated by inserting a small pipe (Figure 3-1). In this method, the test tube rack was divided into three sets, e.g., one control set (A) and the other two sets (B, C) are two replicas of a single treatment, as shown in Figure 3-1. This procedure was repeated many times to collect the required replicas of one single experiment for each plant species.

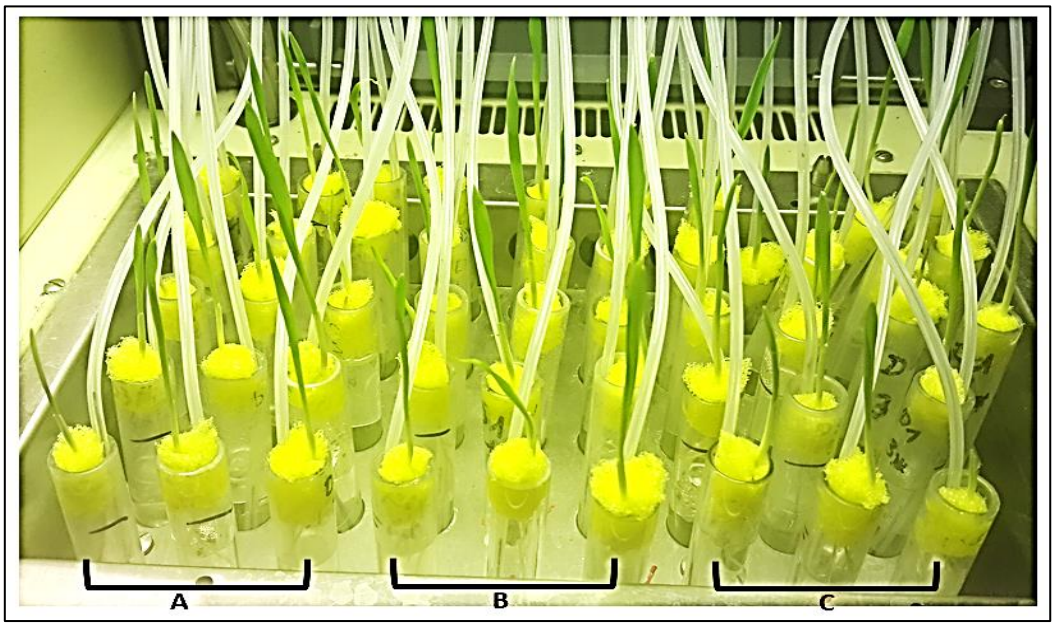

Figure 3-1: First established hydroponic system, where each barley seedling is inserted in a test tube provided with a small pipe for aeration.

Method 2: Every wrapped seedling is fixed in a circular foam sheet (Ø = 10 cm) containing about 20 holes (Ø = ~10 mm) to hold the seedlings (Figure 3-2 A). This sheet was placed firstly on Petri dishes containing 15 ml of Hogland's medium. When the roots reached a length of more than 3 cm, Petri dishes were replaced by small preserving jars containing 50 mL of the culture medium (Figure 3-2 B). Aeration was performed by bubbling air through small pipes inserted inside the media (Figure 3-2 B).

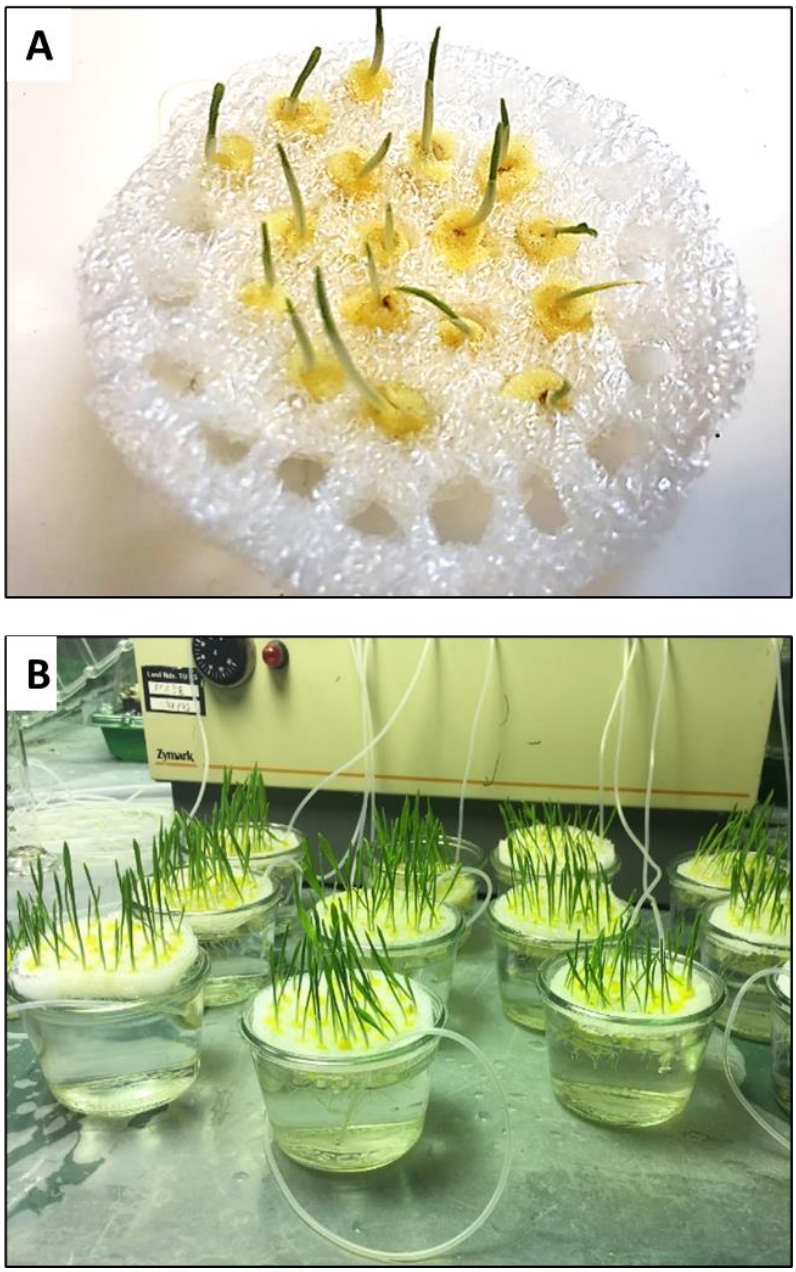

Figure 3-2: Final established hydroponic system, where all barley seedlings of one batch are fixed in a foam sheet (A) and placed in a medium-containing jar aerated by bubbling air through small pipes (B).

3.4 Application of different coumarins

Two weeks after germination, when the seedling roots had reached a length of about 6 cm, the culture medium was exchanged by a new one. Different coumarins, like umbelliferone, esculetin, and esculin were added separately to the medium in a concentration of 200 µg/mL.

In this context, a simple experiment was performed to ensure that the detected coumarins in the shoots were due to their uptake by the roots, not to their ascending on the shoots by the capillary action. In this approach, a piece of tissue paper was wrapped gently around the stem of each seedling for both the control and the treated plants (Figure 3-3).

Figure 3-3: Barley seedlings wrapped gently by tissue papers.

After five days of coumarin application, the tissue pieces were removed, collected, and soaked in a 50 mL Falcon tube containing 20 ml of methanol (99.9%, HPLC grade). The soaked papers were sonicated for 30 minutes using an ultrasonic bath (Bandelin Sonorex), then centrifuged for 10 minutes (Hettich Rotixa/RP centrifuge), and the supernatant was decanted into another tube. The supernatant was evaporated by using a gentle stream of air (Zymark Turbo Vap evaporator) until dried. To prepare the sample for HPLC analysis, the residue was redissolved in 1 mL of 80% methanol.

On the other hand, the shoots of the seedlings were also harvested by cutting them 1-2 cm above the sponge. This procedure guarantees that the harvested plant material had no direct contact with the culture medium via the moist sponge.

Directly after cutting, the harvested shoots were transferred into liquid nitrogen for 5 min. After that, the frozen plants were dried inside the freeze-dryer (Finn-aqua, lyovac GT 2) for 2-3 days to be ready for the extraction procedure.

3.5 Extraction of the dried plant material

The freeze-dried collected plants were ground using a ball mill (Retsch MM200). The plant powders were collected and stored in sample bottles. For extraction, 200 mg of the powdered sample were weighed using an analytical balance (Sartorius analytic) and suspended in 3 mL of 80% methanol. The suspension was sonicated at 50 °C for 30 minutes, then centrifuged (4000 rpm for 10 minutes), and the supernatant was decanted. The pellet was resuspended in 3 mL of 80% methanol. These steps were repeated twice. All the supernatant fractions were pooled together and evaporated using Zymark Turbo Vap evaporator, at 40 °C to dryness. For the HPLC analysis, the samples were redissolved in 1mL of 80% methanol, sonicated, and filtered using an online syringe filter (Spartan, 0.45 μm) inside HPLC vials.

3.6 HPLC analysis

The HPLC (Merck L-6200, Hitachi) analysis was performed using a Nucleosil RP-C18 column (5 μm particle size, L×I.D. 25 cm×3.2 mm, 100A°). The mobile phase was a mixture of A: aqueous acetic acid (1%) and B: acetonitrile, with a gradient mode as following: initial 95% A, 5% B; 20 min 80% A, 20% B; subsequently the ratio was changed as follows: 25 min: 75% A, 25% B; 30 min: 65% A, 35% B; 35 min: 85% A, 15% B; 39 min: 95% A, 5% B; 50 min: 95% A, 5% B. The flow rate was 0.8 mL min^{-1} and the injection volume was 25 μl, while the total run time was 50 minutes. For detection, a Schimadzu fluorescent detector was employed (λex.=350 nm, λem. = 430 nm).

3.7 LC-MS analysis

To confirm and verify the identity of the putative umbelliferone derivatives scopoletin and esculin, high-resolution UPLC-MS analysis was performed using an Acquity UPLC (Waters) with a Kinetex 2.6 μm C18-column (Phenomenex, 100×4.6 mm) with a linear gradient of (A) 0.1% formic acid solution and (B) acetonitrile with 0.1% formic acid at a flow rate of 600 μL/min (initial: 90% A, 10% B; 10.0 min: 10% A, 90% B; 12.0 min: 10% A, 90% B; 12.5 min: 90% A, 10% B; runtime: 15 min). MS and MS/MS analysis were performed on a Q-Tof Premier (Waters) using electrospray ionization (positive ions, 3 kV capillary voltage, 350 °C desolvation temperature, and 15-30 eV collision energy for MS/MS with argon as collision gas).

Using this analysis program, scopoletin showed a retention time of 4.54 min (HR-MS: $C_{10}H_9O_4$ $[M+H]^+$ calc: 193.0501, found: 193.0503). While esculin eluted at 2.86 min (HR-MS: $C_{15}H_{17}O_9$ $[M+H]^+$ calc: 341.0873, found: 341.0872. To confirm the results of the second derivative, the MS/MS analysis of precursor ion m/z=341 resulted in a strong fragment (m/z=179), which corresponds to esculin detection in complex samples.

3.8 Further experimental approaches

3.8.1 Collection of Guttation droplets

In order to collect the Guttation droplets for further investigation, the seedlings of barley and garden cress were grown in a hydroponic system as described in (section 3.3; method 2). Two weeks after germination, when the plants are well grown, the jars of control and treated plants were placed in a big plastic box with a lid. The box is filled by one-tenth of its volume with water and closed. Then, the closed box was wrapped with a blanket during the night, to increase the humidity

in the surrounding atmosphere of the plants. Early in the next morning, the small droplets that formed on the leave tips were collected carefully by a pasture pipette from both the control and treated plants. This procedure was repeated daily for four days to collect adequate amounts, and they were stored in the fridge until analyzed. The collected droplets were then evaporated using a gentle stream of air (Zymark Turbo Vap evaporator), and the residue was redissolved in 1 mL of 80% methanol to be ready for the HPLC analysis.

3.8.2 Application of some enzyme inhibitors

For certain experimental approaches, besides coumarins, the following enzyme inhibitors were also added to the culture medium, as outlined below.

A)-Naproxen (a putative inhibitor of cytochrome P450 enzymes): Seeds of barley and *Lepidium* were germinated and grown, as described in method 2 (section 3.3). When the plants are well grown (two weeks after germination), naproxen was applied in a final concentration of 200 μg/mL to the culture medium simultaneously with umbelliferone. To assess accurately the activity of the inhibitor, an experiment employing seedlings treated only with umbelliferone was also performed at the same time. After five days, the shoots of both plant species were harvested, dried, ground, and extracted to be analyzed by HPLC as described in sections (3.5; 3.6).

B)-N-D-Glucosyl piperidine and N-D-Galactoosyl piperidine (inhibitors of glucosidases): Seeds of barley and *Raphanus* were germinated and grown, as described in method 2 (section 3.3). When the plants are well grown (two weeks after germination), the inhibitors were applied in a final concentration of 200 μg/mL to the media without the addition of coumarin glucoside, i.e., esculin. After several hours, esculin was added to the culture medium of both plants. To assess accurately the activity of the inhibitors, an experiment employing plants treated

only with esculin was done also at the same time. The shoots of all plant species were harvested after five days, dried, ground, and extracted to be analyzed by HPLC as described in sections (3.5; 3.6).

It is worth mentioning that a simple approach was performed to confirm the activity of apoplastic glucosidases. Barley seedlings were cultivated as described in method 2 (section 3.3). After two weeks of germination, when the seedlings are well grown, a tiny amount (~ 10 mg) of 4-Methylumbelliferyl-β-D- glucoside was added to the culture medium. The appearance of a blue fluorescence through the medium is considered as proof for the glucosidase activity in the medium.

3.8.3 Incubation of excised leaves and roots with umbelliferone

In the following experiment, barley and *Lepidium* were cultivated in a hydroponic system as described in method 2 (section 3.3). After two weeks of germination, when the plants are well grown, their leaves and roots were excised.

The cut leaves and roots were incubated separately inside Petri dishes containing 20 mL of umbelliferone (Figure 3-4A; B, respectively). After five days, these roots and leaves were washed twice with distilled water to remove any adhered residues of umbelliferone, dried by paper towel and dipped in liquid nitrogen, then in the freeze dryer, ground, and extracted to be analyzed by HPLC as described in sections (3.5; 3.6). As a control, the cut leaves and roots of the same plant species were handled in the same manner, however, they were incubated only in a culture medium without umbelliferone.

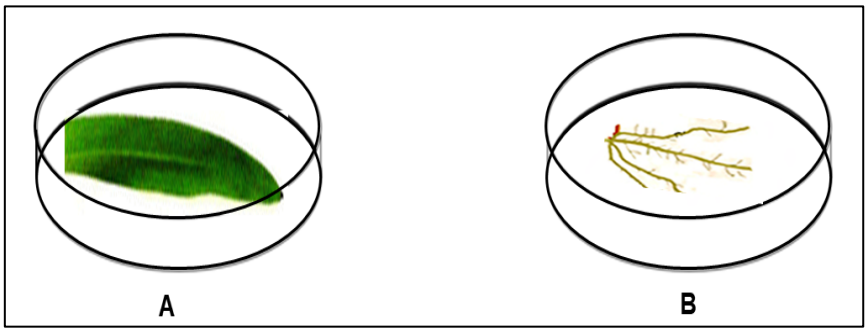

Figure 3-4: Excised leaves (A) and roots (B) are incubated with umbelliferone in Petri dishes.

In another experimental approach, only excised barley leaves were employed, however in a slightly different setup: The cut barley leaves were incubated in umbelliferone-containing medium into two different positions; an upright position where the cut ends of the leaves were immersed in the medium (Figure 3-5A), and upside-down position where just the tips of the leaves were soaked in the medium (Figure 3-5B). After five days, these leaves were washed twice with distilled water to remove any adhered residues of umbelliferone, dried by paper towel, and dipped in liquid nitrogen, then in the freeze dryer, ground, and extracted to be analyzed by HPLC as described in sections (3.5; 3.6). As a control, the cut leaves of barley were handled in the same manner, however, they were incubated only in a culture medium without umbelliferone.

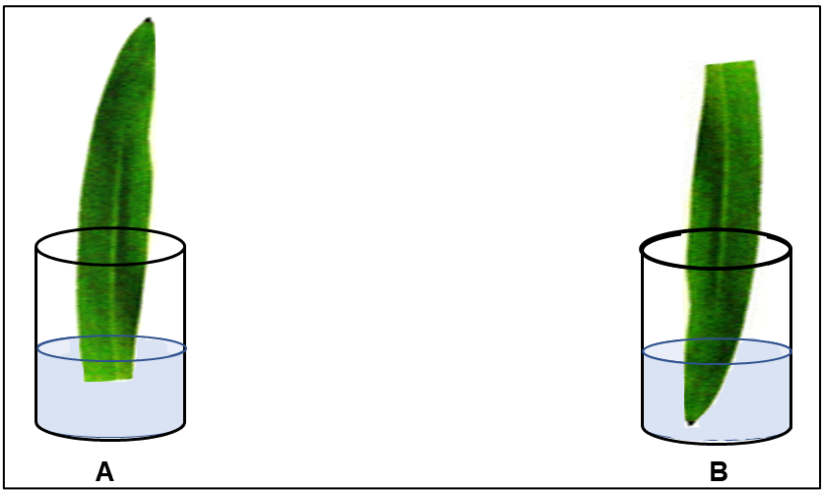

Figure 3-5: Excised barley leaves are incubated with umbelliferone, either in an upright position (A) or in an upside-down position (B).

Chapter 4: Results

4.1 Establishing and optimization of a suitable hydroponic system

In order to thoroughly investigate the uptake of a certain compound by plants, a suitable and efficient system that allows the required variation of experimental parameters has to be established and optimized. Therefore, a convenient hydroponic system was developed and optimized to finally achieve the requirements.

In the first trial, various sizes and shapes of test tubes were used to host a single seedling (Figure 4-1). However, this approach was time-consuming, since the second replica of the same experiment is done after more than one week when the first one is finished. The necessary aeration required was realized by bubbling air through small tubes. Unfortunately, the airflow could not be regulated properly to ensure identical aeration in all tubes.

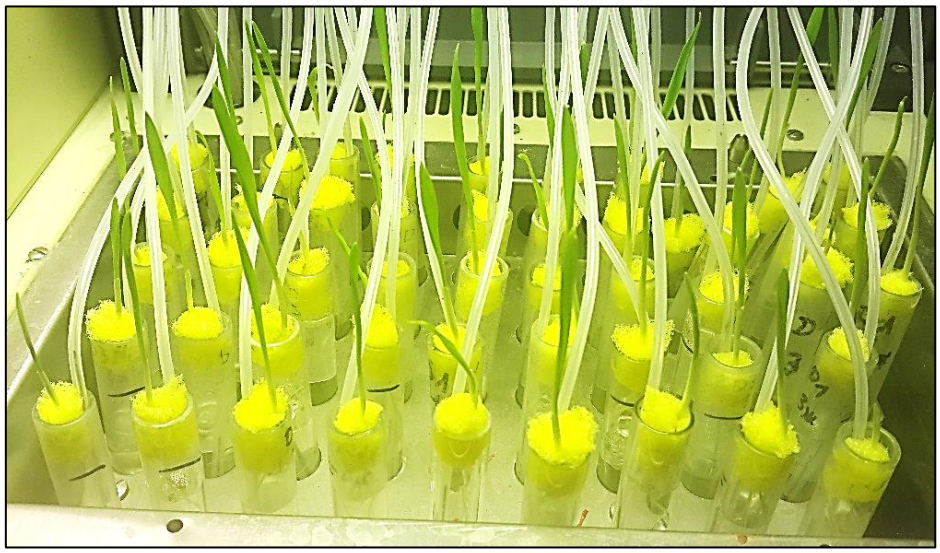

Figure 4-1: Barley seedlings wrapped by sponge and cultivated in test tubes containing Hogland's medium. Aeration was performed by the small pipes (the first trial in establishing the hydroponic system).

Consequently, the conditions for the seedlings of one batch had not been really identical. Therefore, all seedlings of one batch should be cultivated in the same

medium. This, however, requires an appropriate fixation and positioning system. After several attempts, an adequate system was established. In a circular foam sheet (Ø = 10 cm) about 20 holes (Ø = ~10 mm) had been punched to host the seedlings. Each seedling was wrapped separately by a small piece of sponge before insertion into the holes. This procedure ensured optimal fixation without any damage to the seedlings.

At first, the sheet was placed on a petri dish containing 15 mL Hoagland solution. As a result, the roots of the 20 seedlings were immersed in the culture medium, whereas the shoots had no direct contact with it. Aeration was performed by bubbling air through a small tube. When the roots had achieved a length of more than 3 cm the petri dish was exchanged by a small preserving jar containing 50 mL of the culture medium (Figure 4-2).

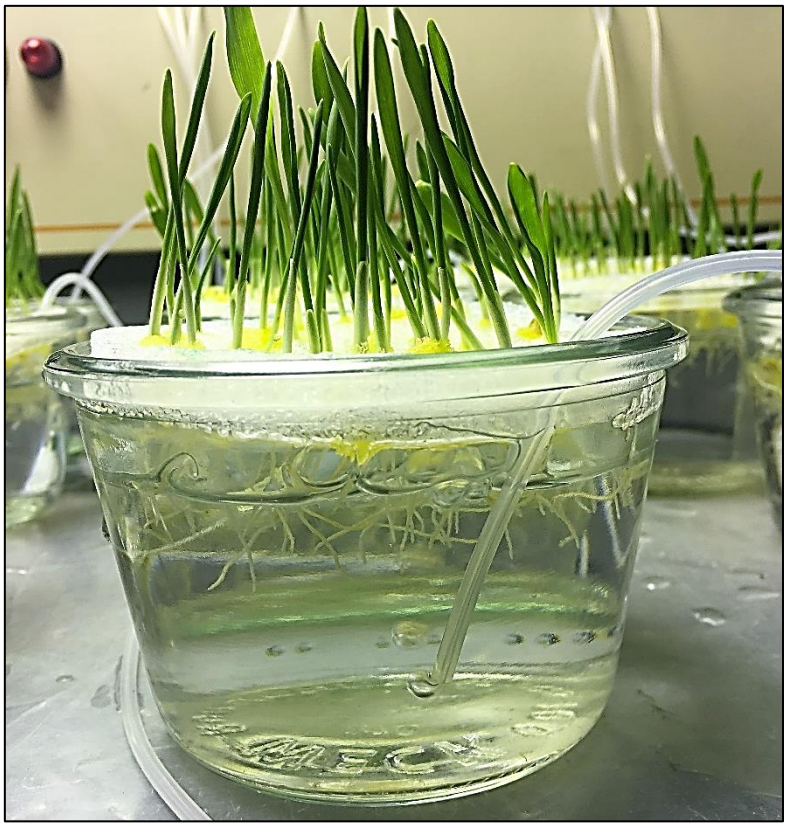

Figure 4-2: Barley seedlings fixed in foam sheet and placed on a jar containing Hogland's medium. Aeration was performed by the small pipes.

Aeration was achieved by bubbling sterile air through small pipes. Details are displayed in the "Materials and methods" chapter (section 3.3).

It is important to mention that this technique enables the roots to grow within the culture medium, whereas a direct contact of the aerial parts with the medium - and thus with the substances applied - was excluded. However, to foreclose that the culture medium is ascending to the shoots by capillary action, a related inspection had to be performed.

For this, small pieces of tissue paper were wrapped around the growing seedlings, before cultivating them in a medium containing umbelliferone (Figure 4-3). After five days of cultivation, in addition to the analyses of the areal parts, also the pieces of tissue paper were extracted and analyzed by HPLC.

Figure 4-3: Growing seedlings wrapped by small pieces of tissue papers, before cultivating them in a medium containing umbelliferone.

Indeed, the tissues wrapped around the seedlings fed with umbelliferone contained small amounts of umbelliferone (Figure 4-4). But, also in the tissues wrapped around the control seedlings, umbelliferone was present. Obviously, a small share of the endogenous umbelliferone present in the control plants diffused into tissue paper. Analogously, the elevated umbelliferone concentration in the leaves of the seedlings grown in the umbelliferone-containing media resulted in a

slightly enhanced diffusion into the paper. In contrast, in case of a direct contact of the tissue with the umbelliferone-containing medium, the concentration of the coumarin is far higher (Figure 4-4). These data clearly show that the umbelliferone detected in the leaves indeed is due to a translocation within the seedlings, an apoplastic transfer by diffusion via capillary forces can be excluded.

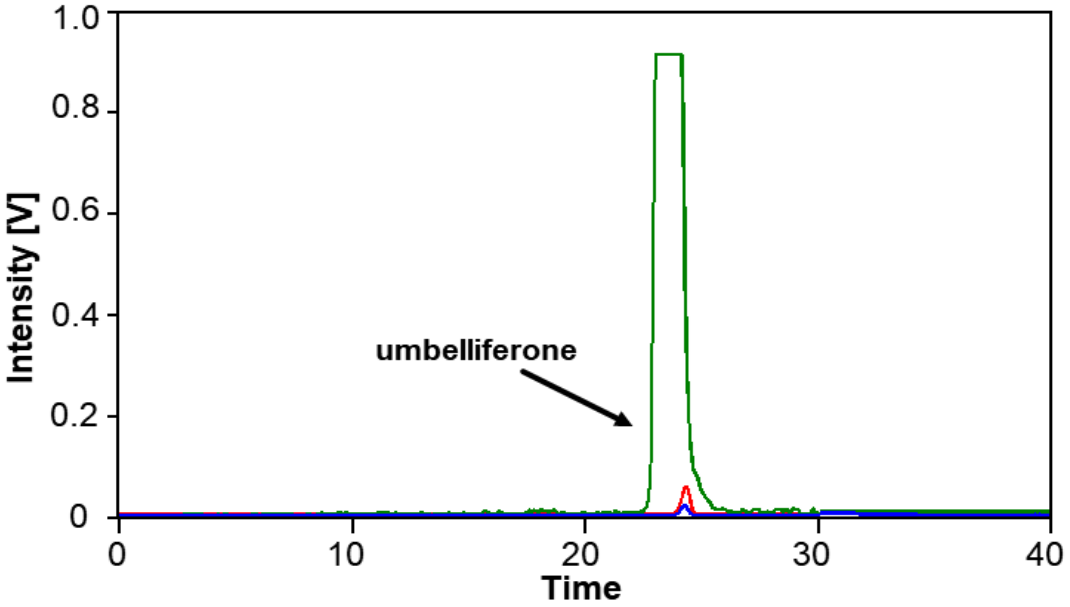

Figure 4-4: Detection of umbelliferone in the tissue papers. The HPLC chromatogram of the tissue papers from the control plants is displayed in blue, this of the treated plants is given in red, and the chromatogram of the tissue papers in direct contact with the medium is in green (diluted four times).

In consequence, these conditions signify, that the umbelliferone which is – in comparison to the controls - additionally detectable in the barley seedlings grown in umbelliferone-containing media, is due to an uptake by their roots and a subsequent translocation into the shoots. Accordingly, this hydroponic system was used for the investigation of umbelliferone uptake in seedlings of five different plant species, i.e., barley (*Hordeum vulgare L.*), radish (*Raphanus sativus L.*), pea (*Pisum sativum L.*), flax (*Linum usitatissimum L.*), and garden cress (*Lepidium sativum L.*).

4.2 Uptake and modification of umbelliferone

As outlined in the introduction, because of their strong fluorescence, the employment of coumarins seemed to be very advantageous to elucidate uptake and putative modification of natural products in the acceptor plants. However, phenolic compounds exhibiting several hydroxyl groups, especially catecholic ones, e.g., esculetin and esculin are oxidized immediately (Appendix, Figure A-1). Consequently, the relatively stable umbelliferone (Figure 1-1, page 3) was chosen as an appropriate model substance to study its uptake and modification within different acceptor plants. Yet, in order to avoid misinterpretations due to putative typical-specific characteristics, seedlings of five different plant species, i.e., flax (*Linum usitatissimum L.*), radish (*Raphanus sativus L.*), pea (*Pisum sativum L.*), barley (*Hordeum vulgare L.*), and garden cress (*Lepidium sativum L.*) had been employed. Accordingly, the hydroponic system described above was used to apply the coumarins to the roots of the various seedlings.

After one week of culturing, in the leaves of all tested species, large amounts of umbelliferone were present, when the seedlings were grown in umbelliferone-containing media. As outlined above, any direct contact of medium with the shoots can be excluded. Thus, the occurrence of umbelliferone in the leaves of all five experimental plants displays that this coumarin indeed is taken up by their roots from the medium and subsequently translocated into the leaves. The identity of umbelliferone was confirmed by employing an authentic standard. In addition to umbelliferone in the leaves of several plant species, additional peaks had been detected in response to the umbelliferone application. The different reactions of the certain seedlings and the corresponding coumarin patterns are outlined in the figures below.

In the case of *Linum*, *Pisum*, and *Raphanus*, the only difference between control and treated plants is given by the enormous accumulation of umbelliferone. Thus, this

coumarin is just taken up from the medium by the plant roots and translocated to the aerial parts (Figure 4-5; 4-6; 4-7).

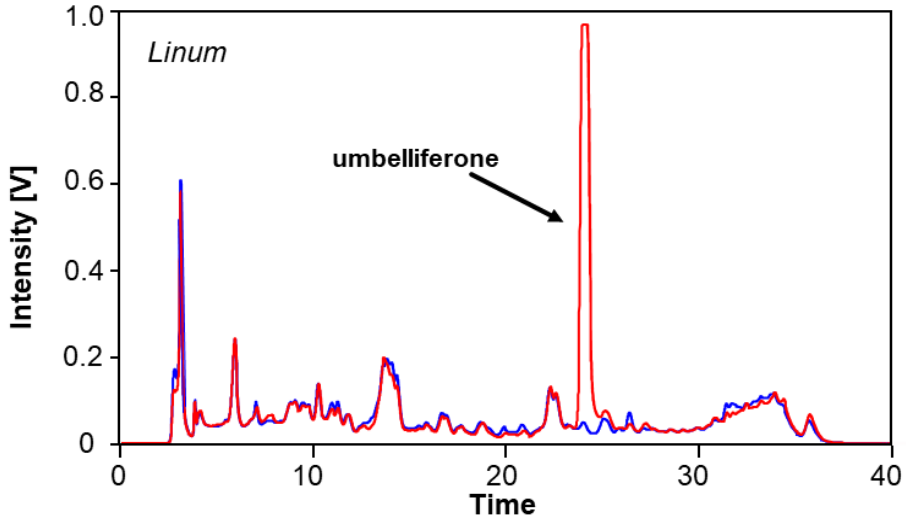

Figure 4-5: Uptake of umbelliferone by flax seedlings (*Linum usitatissimum*). The HPLC chromatogram of the extract from the control plants is displayed in blue, this of the treated plants is given in red.

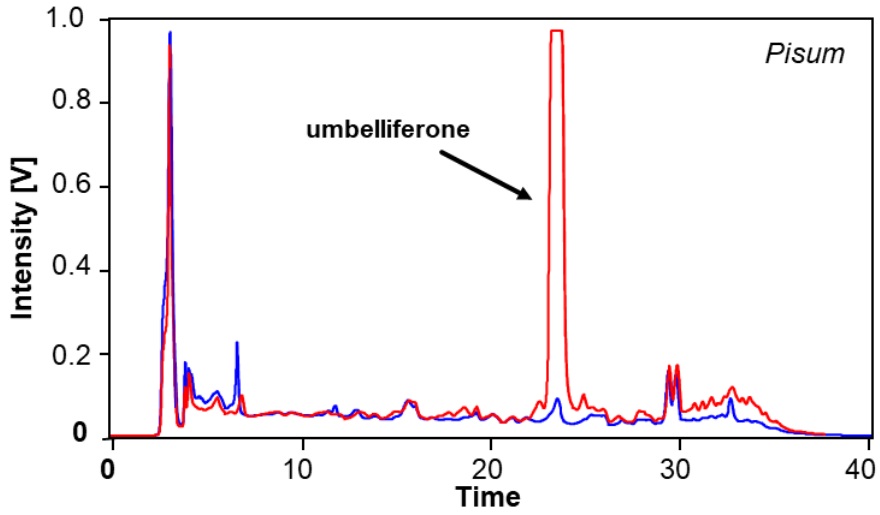

Figure 4-6: Uptake of umbelliferone by pea seedlings (*Pisum sativum*). The HPLC chromatogram of the extract from the control plants is displayed in blue, this of the treated plants is given in red.

In this context, it has to be taken into consideration that in the case of *Raphanus* seedlings, at the first glance, the chromatogram seems to be very complex (Figure 4-7), due to the occurrence of high concentrations of various hydroxycinnamic acids (Stöhr and Herrmann, 1975). Nonetheless, the detailed comparison of the

chromatograms unequivocally shows, that also the *Raphanus* seedlings have taken up umbelliferone and accumulated it to a high extent.

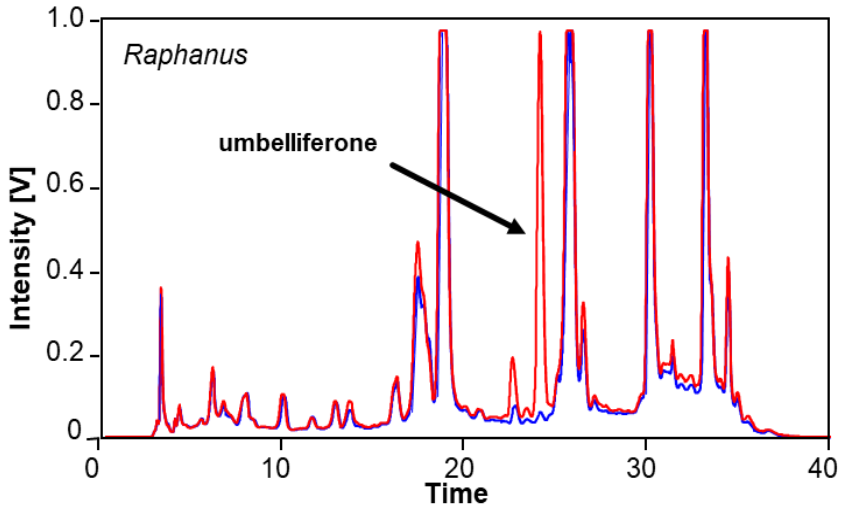

Figure 4-7: Uptake of umbelliferone by radish seedlings (*Raphanus sativus*). The HPLC chromatogram of the extract from the control plants is displayed in blue, this of the treated plants is given in red.

In the leaves of barley, small amounts of umbelliferone are already naturally present, and in garden cress, its endogenous concentration is really high (Figure 4-8; 4-10), respectively. In both cases, the cultivation in umbelliferone-containing media massively enhances the umbelliferone concentration in the leaves, verifying that also in the seedlings of these species the coumarin is taken up and accumulated in the leaves. In contrast to the seedlings of the first three species, in barley and garden cress several additional peaks could be detected in the seedlings treated with umbelliferone.

In the case of barley, just one further compound was generated (peak 1, Figure 4-8).

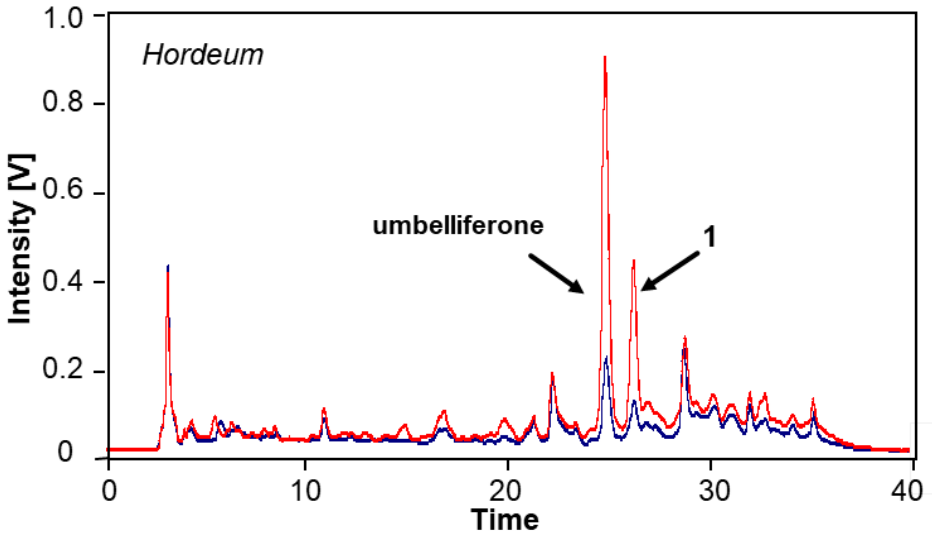

Figure 4-8: Uptake and modification of umbelliferone by barley (*Hordeum vulgare*). The corresponding HPLC chromatogram of the extract from the control plants is displayed in blue, this of the treated plants is given in red.

The comparison of its retention time with those of various coumarins suggested that this substance is scopoletin. It was isolated and further analyzed by LC-MS/MS. The comparison of the related data with those of an authentic scopoletin standard unequivocally verified that compound **1** indeed is scopoletin (Appendix, Figure A-2). Accordingly, it has to be deduced that a quota of the umbelliferone taken up by the barley roots is hydroxylated and methylated to yield scopoletin (Figure 4-9).

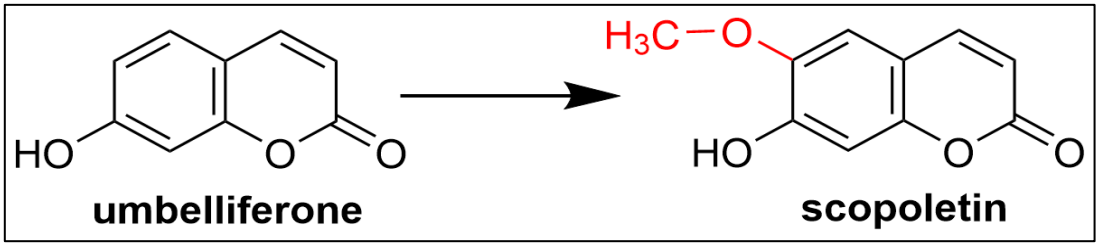

Figure 4-9: Umbelliferone taken up by barley (*Hordeum vulgare*) seedlings is converted to scopoletin.

In case of garden cress, the chromatogram of the leaves extracts from seedlings cultivated in umbelliferone-containing media is very complex compared to that of the control plants (Figure 4-10). Apart from the strong increase in the

umbelliferone concentration mentioned above, in the treated plants, various other substances were produced, i.e, **2**, **3**, and **4**. Moreover, esculetin, which is originally present in the garden cress control plants, is slightly enhanced. In contrast, the concentration of scopoletin, representing the major genuine coumarin of garden cress that is accumulated to a very high extent, seems to be not altered by the umbelliferone treatment (Figure 4-10).

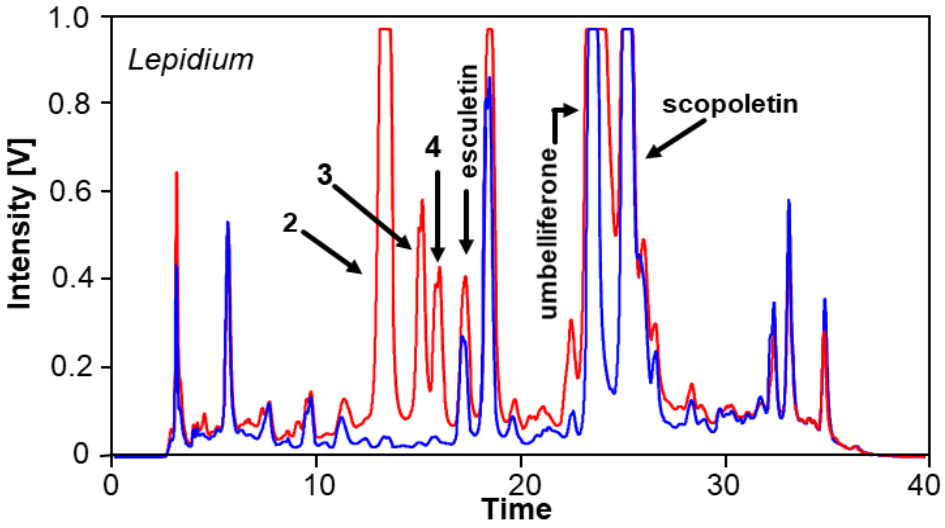

Figure 4-10: Uptake and modification of umbelliferone by garden cress (*Lepidium sativum*). The corresponding HPLC chromatogram of the extract from the control plants is displayed in blue, this of the treated plants is given in red.

The comparison of the retention time of substance **2** with those of the coumarin standards suggested that this substance is esculin. Substance **2** was isolated and further analyzed by LC-MS/MS. The comparison of the related data with those of the authentic esculin standard unequivocally verified that compound **2** indeed is esculin (Appendix, Figure A-3). Accordingly, it can be deduced that a quota of the umbelliferone taken up by the roots of the garden cress seedlings is hydroxylated and then glucosylated to yield esculin within the acceptor plant (Figure 4-11).

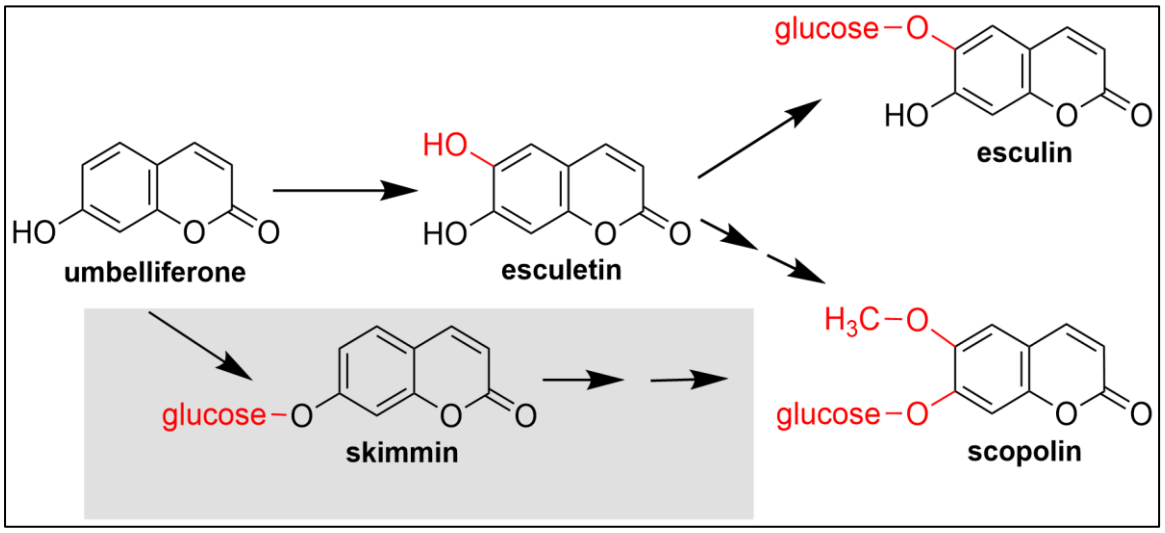

Figure 4-11: Umbelliferone taken up by garden cress (*Lepidium sativum*) seedlings is converted to the glucosides esculin and scopolin, where this conversion is proposed by different pathways. The pathway highlighted in grey is less likely to occur.

By comparing the retention time of substance **4** with the various coumarin standards, it turned out that it represents scopolin. In contrast to the generation of esculin, where the order of reactions is clearly predetermined, i.e., first hydroxylation and subsequent glucosylation, in the case of the scopolin production, there are various possibilities (Figure 4-11). Indeed, based on the occurrence of high amounts of scopoletin in garden cress, it seems to be reasonable that esculetin is methylated to yield scopoletin, which subsequently is glucosylated to scopolin. However, then the question arises, why no scopolin is present in the control plants. Alternatively, umbelliferone might be glucosylated to yield skimmin which subsequently might be hydroxylated and methylated. However, as no skimmin could be detected in the treated plants, this option seems to be very unlikely. The situation becomes even more complicated when considering the occurrence of substance **3**. Based on its fluorescence properties, this compound also seems to represent glucoside of a hydroxylated coumarin, which presumably might be isoscopolin, but – up to now – its structure could not be elucidated. Accordingly, it cannot be stated whether or not this compound is an

intermediate in the course of the scopolin generation. This complex issue is outlined and explained in detail in the "Discussion" chapter (section 5.3.1).

Although not all details are elucidated so far, it can be stated unequivocally, that large shares of the umbelliferone imported into barley and garden cress are modified. Whereas in barley, scopoletin represents the major product of modification, in garden cress the lion's share of the imported umbelliferone is converted to esculin. These results for the first time verify that – as postulated – also natural products taken up by acceptor plants are modified. As it is reported for numerous xenobiotics, also the imported coumarins are hydroxylated, methylated, and glucosylated.

It has to be noted that both species, garden cress and barley, endogenously contain umbelliferone. Accordingly, it could be argued that the umbelliferone taken up is integrated within the metabolic reactions and pathways in analogy to the endogenous one. This might be the case in barley, where – apart from umbelliferone – small amounts of scopoletin are also present in the control plants. In case of an enhancement of the internal umbelliferone concentration by external application, also the concentration of scopoletin is elevated due to an increase in the conversion of umbelliferone to scopoletin. In contrast, the situation in garden cress is far more confusing. Although large amounts of umbelliferone and scopoletin are inherently present, no esculin or scopolin is present in the control plants. However, when umbelliferone is taken up, a major share of this coumarin is converted to esculin and scopolin. The question arises, why the endogenous umbelliferone is not converted to the corresponding glucosides. Indeed, a putative explanation for this phenomenon could be the fact that the imported umbelliferone is temporarily localized in a different compartment than the endogenous one. Yet, it has to be considered that coumarins easily diffuse through membranes (see above, Figure 4-4), and thus, differences in cellular localization can be ruled out as

an explanation for the observed variations. In principle, there are two explanations for the differences in the fate of endogenous and imported umbelliferone: either the application of umbelliferone changes the activity of relevant enzymes, or there are spatial differences in the enzyme configuration. Accordingly, it is of special interest to elucidate, where the conversion of the imported umbelliferone takes place, i.e., in the roots or the leaves.

In summary, the relative concentrations of the imported umbelliferone and its modified products in seedlings of the various species are compiled in Table 1.

Table 4-1: Concentration of umbelliferone and its derivatives in the acceptor plants.

Acceptor Plants	Concentration (µg/g d.w)		
	umbelliferone	scopoletin	esculin
H. vulgare, control	0.22 ± 0.15	0.82 ± 0.09	-
H. vulgare + umbelliferone	62.7 ± 4.1	8.2 ± 1.3	-
L. sativum - control	22.05 ± 0.85	39.5 ± 1.2	0.23 ± 0.06
L. sativum + umbelliferone	41.6 ± 2.6	49.0 ± 2.1	18.3 ± 6.2
L. usitatissimum - control	1.10 ± 0.51	-	-
L. usitatissimum + umbelliferone	58.6 ± 3.1	-	-
P. sativum - control	2.0 ± 0.4	-	-
P. sativum + umbelliferone	41.5 ± 5.2	-	-
R. sativus - control	0.04 ± 0.02	-	-
R. sativus + umbelliferone	14.1 ± 1.1	-	-

The concentration of the various coumarins has been quantified by HPLC. In all cases, the concentrations represent the mean values of three independent experiments applying about 15 to 20 seedlings for each approach. d.w: dry weight

4.2.1 Translocation of the imported umbelliferone

4.2.1.1 Analysis of guttation droplets

The umbelliferone, which is taken up by the roots, subsequently is translocated into the leaves of the acceptor plants. In principle, there are two options for the transport processes in plants, either via phloem or via xylem. A high accumulation of the imported umbelliferone in the leaves suggests that the coumarin is translocated via the xylem. A corresponding situation was reported for nicotine (Selmar et al., 2015a, b; Weidner et al., 2005) and pyrrolizidine alkaloids taken up by various acceptor plants (Nowak 2017) as well as for xenobiotics (Trapp and Legind, 2011).

In order to verify that the imported umbelliferone also is translocated analogously, the xylem sap has to be analyzed. The most convenient approach for such an investigation could be realized by collecting and analyzing guttation droplets. Accordingly, garden cress and barley plants, which had been cultivated in umbelliferone-containing media, were urged to produce guttation droplets by massively increasing the humidity ("Material and methods" chapter, section 3.8.1). As a result, on the tips or edges of the barley leaves typical exudation droplets were formed (Fig. 4-12).

For each trial, i.e., controls or seedlings cultivated in a hydroponic system containing umbelliferone, about 500 guttation droplets were collected from the tip of the leaves, either from barley or from garden cress seedlings.

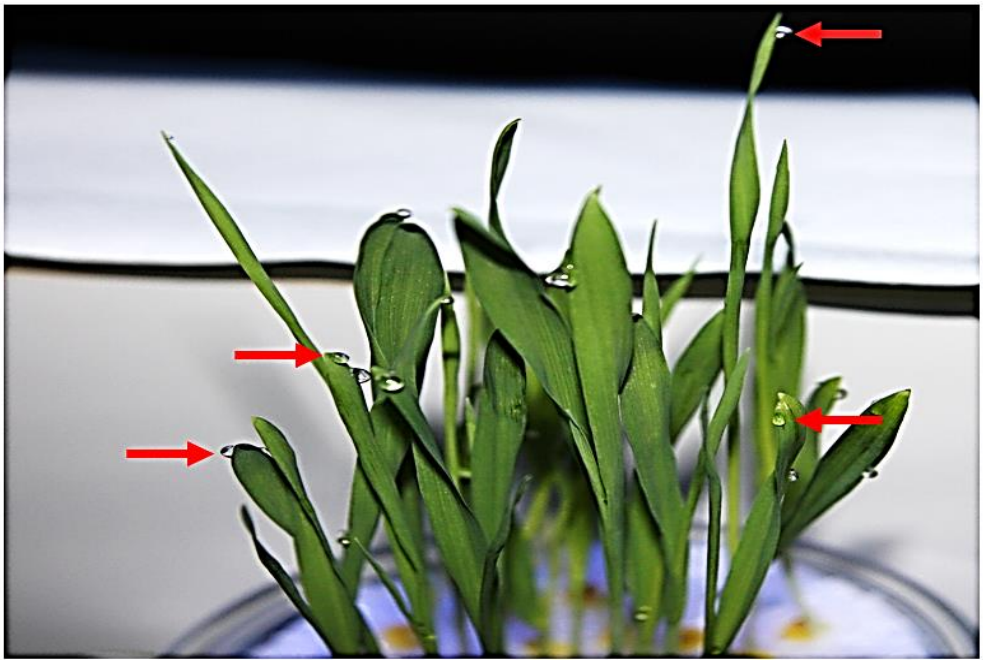

Figure 4-12: Guttation droplets on barley leaves are indicated by the red arrows.

When the guttation droplets are irradiated with a UV lamp, they nicely fluoresce blue (Figure 4-13), pointing to the occurrence of coumarins.

Figure 4-13: Guttation droplets of barley treated with umbelliferone are fluorescing blue.

The HPLC analyses of the guttation droplets from the barley control plants reveal the occurrence of traces of umbelliferone (Figure 4-14). This finding is in accordance with the fact that the coumarins present in barley, predominantly umbelliferone, easily diffuse across the biomembranes (Figure 4-4). Consequently, umbelliferone also is present in the apoplasmic space, and thus - even without any occurrence in the xylem - traces of this compound will be found in the guttation droplets. However, its concentration in the guttation droplets of the seedlings, which have been grown on umbelliferone-containing media, is far higher (Figure 4-14). Moreover, scopoletin is found in the droplets, too.

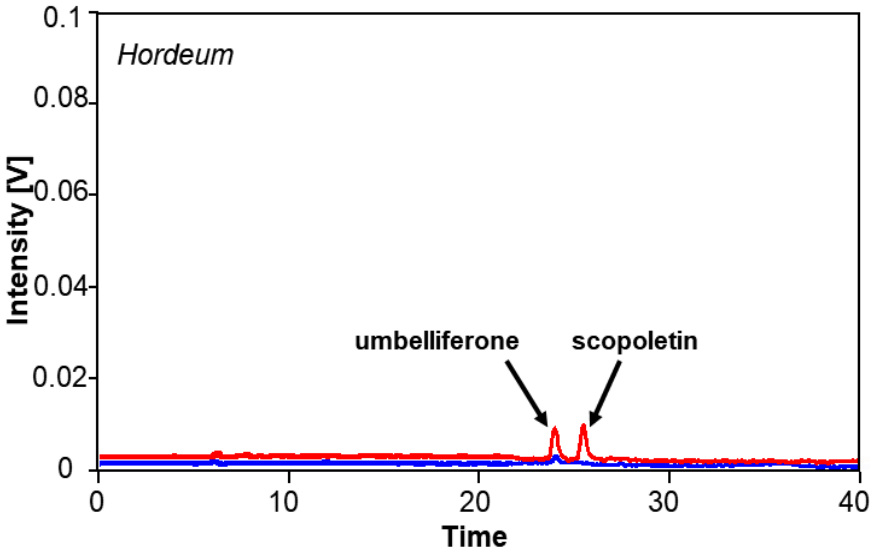

Figure 4-14: Analyses of Guttation droplets of barley (*Hordeum vulgare*). The corresponding HPLC chromatogram of the guttation droplets from the control plants is displayed in blue, this of the treated plants is given in red.

These findings underline the assumption that umbelliferone, and maybe also scopoletin, are translocated from the roots to the shoots via xylem. The presence of the modified product, i.e., scopoletin, might delude that the modification of the imported umbelliferone may already take place in the roots. However, in this case, the ratio of umbelliferone to scopoletin should be the same in all tissues, including the guttation droplets. But, whereas the concentration of scopoletin in the leaves is far lower than that of umbelliferone (Figure 4-8), in the guttation droplets their

concentrations are the same. Considering the physicochemical properties of coumarins, which enables to simply pass biomembranes, the enhanced ratio of scopoletin to umbelliferone in the guttation droplets points to the fact that scopoletin – at least in parts – already is modified in the roots and in addition to umbelliferone, translocated via xylem in the leaves.

In the case of garden cress, the guttation droplets of the control plants show only traces of scopoletin, and no umbelliferone (Figure 4-15). This is consistent with their relative concentrations in the control plants, i.e., the slightly higher concentration of scopoletin in comparison to that of umbelliferone (Figure 4-10).

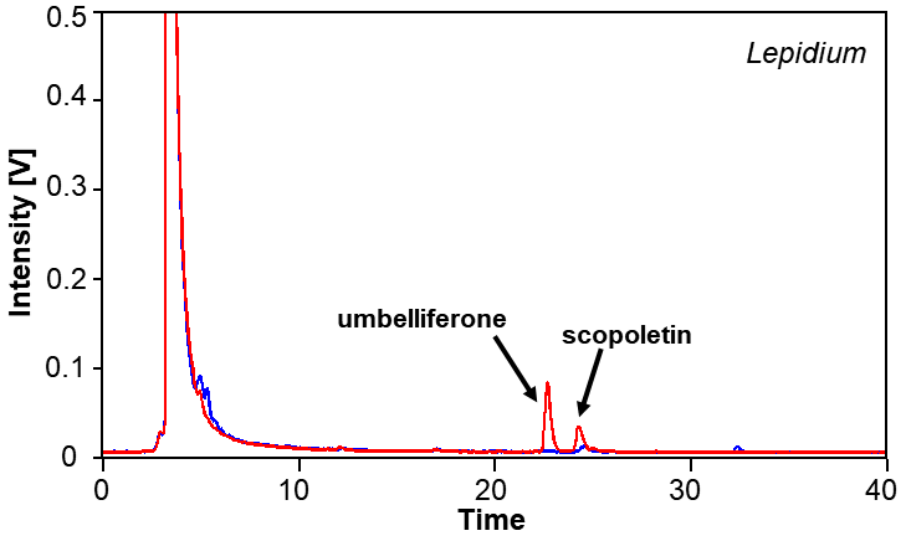

Figure 4-15: Analyses of Guttation droplets of garden cress (*Lepidium sativum*). The corresponding HPLC chromatogram of the guttation droplets from the control plants is displayed in blue, this of the treated plants is given in red.

In the guttation droplets of the *Lepidium* seedlings grown in umbelliferone-containing medium, the concentration of both scopoletin and umbelliferone is strongly enhanced. Yet, in this case, the umbelliferone concentration is higher than that of scopoletin (Figure 4-15), a fact which is in accordance with the finding that also in the related leaves the umbelliferone concentration is higher than that of scopoletin. The massive enhancement of both coumarins in the guttation droplets of treated seedlings confirms a translocation from the roots via the xylem to the

shoots. However, due to the nearly concurrent ratios of the concentrations of both coumarins, no deduction with respect to the site of conversion could be made.

With respect to the glucosylated derivatives, the situation is different. In contrast to the leaves of the garden cress seedlings grown in umbelliferone-containing medium (Figure 4-10), no coumarin glucosides i.e., esculin and scopolin, are present in the related guttation droplets (Figure 4-15). In case of a glucosylation in the roots and subsequent translocation into the leaves, these glucosides should be detectable in the guttation droplets. Accordingly, their absence confirms that their production, i.e., the glucosylation, occurs in the leaves. As their physicochemical properties hinder the diffusion across membranes, these compounds will be trapped within the cells and no diffusion into the apoplasmic space could occur.

4.2.1.2 Nature of transported compounds – site of modification

To get further clues on the site of the conversion of umbelliferone to its derivatives, umbelliferone was applied to the isolated putative organs of modification, i.e., roots and shoots, respectively. Accordingly, umbelliferone was incubated with either excised leaves or excised roots of both, barley and *Lepidium* seedlings.

The incubation of cut leaves or roots of *Lepidium* with umbelliferone resulted in a massive accumulation of this coumarin, i.e., the endogenous concentration of umbelliferone was enhanced tremendously. Moreover, the derivatives of the umbelliferone were produced in both, the cut leaves (Figure 4-16) and the cut roots (Figure 4-17); just like in the entire *Lepidium* seedlings, which had been fed with umbelliferone (Figure 4-10). However, the concentration of the derivatized compounds in the cut leaves is far higher than that of the cut roots. As the esculin was not detected in the guttation droplets, and thus, its translocation via xylem could be excluded (see "Discussion" chapter, sections 5.2.2; 5.3.2), the esculin and the other glucosidic derivatives should be restrained in the roots. In consequence,

the esculin accumulated in the leaves should result from the imported umbelliferone, which is biotransformed within the leaves, as outlined by the direct incubation of cut leaves with umbelliferone.

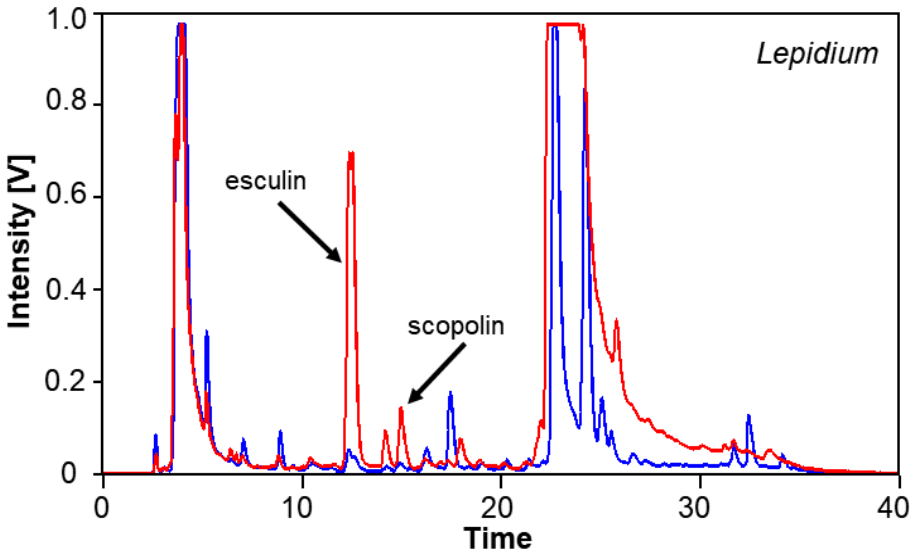

Figure 4-16: Uptake and modification of umbelliferone by excised leaves of garden cress (*Lepidium sativum*). The corresponding HPLC chromatogram of the extract from the control leaves is displayed in blue, this of the treated leaves is given in red.

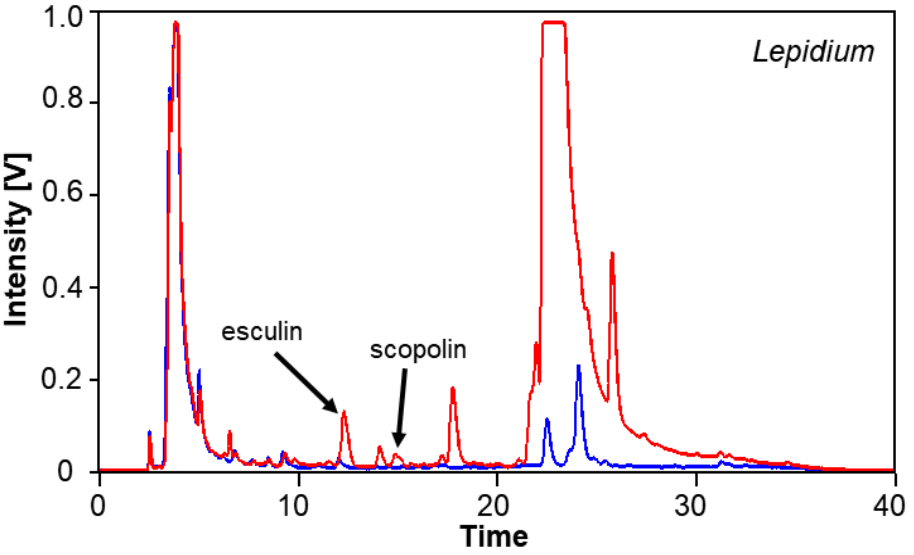

Figure 4-17: Uptake and modification of umbelliferone by excised roots of garden cress (*Lepidium sativum*). The corresponding HPLC chromatogram of the extract from the control roots is displayed in blue, this of the treated roots is given in red.

In the barley, the roots efficiently took up the umbelliferone, but no scopoletin was detectable (Figure 4-18).

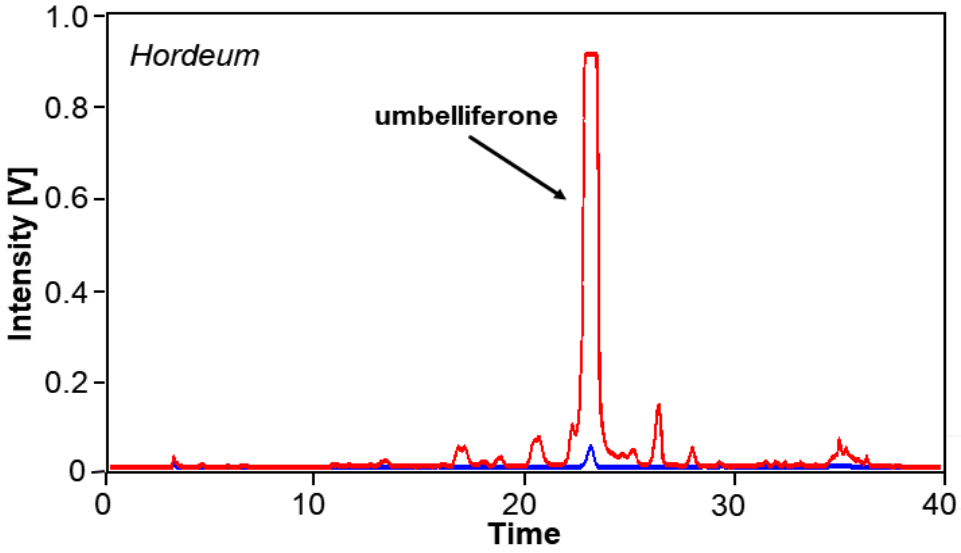

Figure 4-18: Uptake and modification of umbelliferone by excised barley (*Hordeum vulgare*) roots. The corresponding HPLC chromatogram of the extract from the control roots is displayed in blue, this of the treated roots is given in red.

In contrast, in the leaves incubated with umbelliferone, apart from the genuine coumarin, also its methylated derivative scopoletin was present (Figure 4-19). Surprisingly, in contrast to the experiments, in which umbelliferone was applied to the entire seedlings (Figure 4-8), in the excised leaves, also very high amounts of the glucosidic coumarins, i.e., scopolin and esculin are present (Figure 4-19).

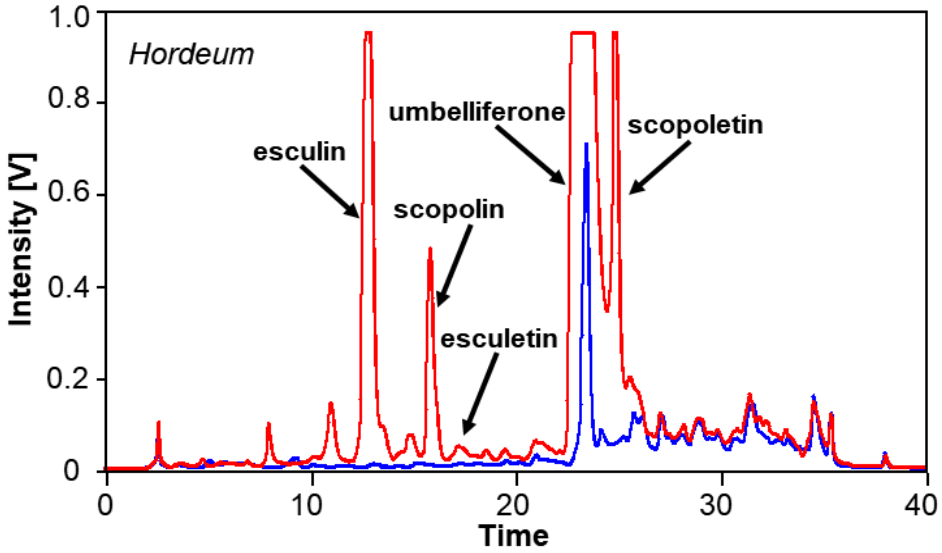

Figure 4-19: Uptake and modification of umbelliferone by excised barley (*Hordeum vulgare*) leaves. The corresponding HPLC chromatogram of the extract from the control leaves is displayed in blue, this of the treated leaves is given in red.

Since the pattern of the modified products significantly differs between the barley seedlings and the excised leaves, i.e., the glucosidic coumarins are only detectable in the excised leaves incubated with umbelliferone, the question arises why are the coumarins not glucosylated in the entire seedlings, although they are present in high concentrations in the leaves, and why is the "esculetin-glucoside" mainly produced.

The main difference between both approaches is the translocation route. In case of the whole seedlings, translocation into the leaves occurs only via the veins, putatively via the xylem (Figure 4-20, A). Accordingly, there will be a concentration gradient from interior to exterior areas. In contrast, the gradient is reversed, when the leaves are immersed in coumarin-containing solutions, and the highest concentrations should be present in the epidermal layers (Figure 4-20, B).

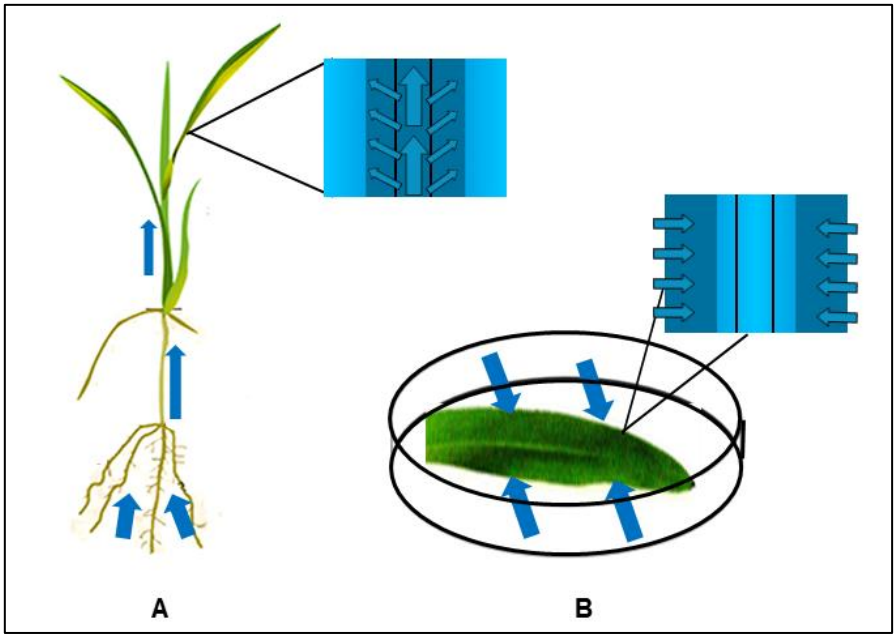

Figure 4-20: Differences in the translocation of umbelliferone from the medium into the leaves. In the case of whole seedlings (A) transfer is performed via xylem, whereas in the cut leaves, umbelliferone diffuses across the entire leaf surface (B).

In order to investigate, whether or not this spatial difference is responsible for the observed differences in the glucosylation pattern, a further approach of umbelliferone application was performed mimicking both options of spatial

localization. For this, some leaves were incubated in an upright position, where only the cut ends were immersed in medium (Figure 4-21, A). This approach ensured that the uptake exclusively takes place at the incision and the further translocation into the leaf blade proceeds via xylem, analogously to the translocation in whole seedlings. Alternatively, leaves were placed upside down (Figure 4-21, B), in this case, just the tips of the leave blades were immersed in the culture medium containing umbelliferone. This approach corresponds to the experiment, in which the entire leaves had been immersed and the uptake mainly occurred via diffusion across the leaf surface.

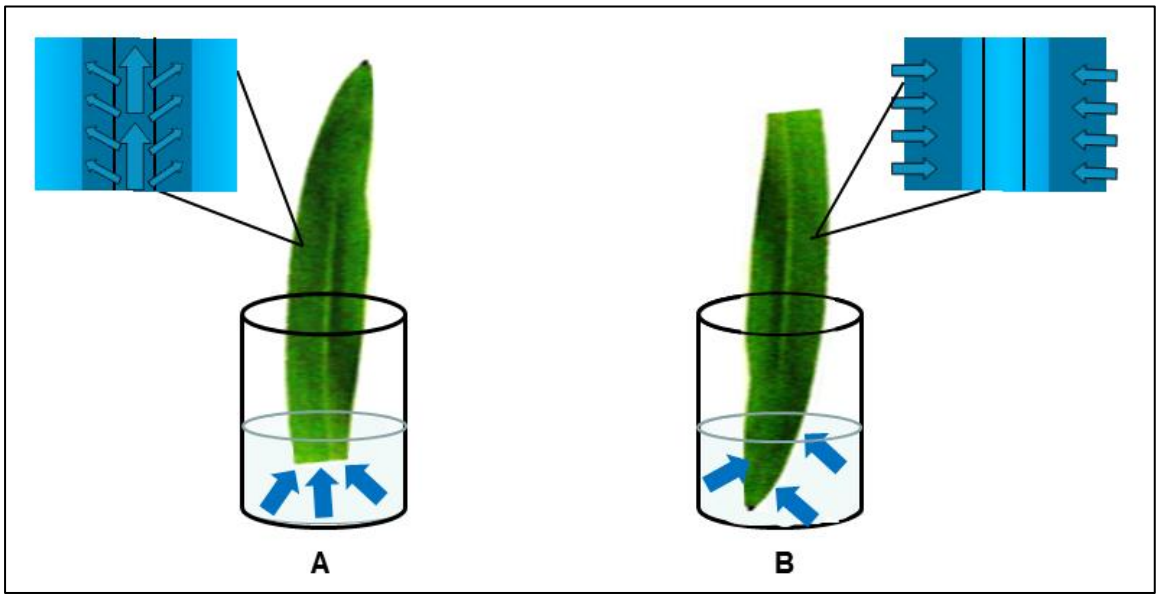

Figure 4-21: Differences in the translocation of umbelliferone from the medium into the leaf blade either via xylem in the upright position (A) or direct diffusion across the leaf surface (B).

Surprisingly, in both cases, the same patterns of coumarins were noticed (Figure 4-22; 4-23): apart from umbelliferone and scopoletin, also the glucosidic derivatives esculin and scopolin formed.

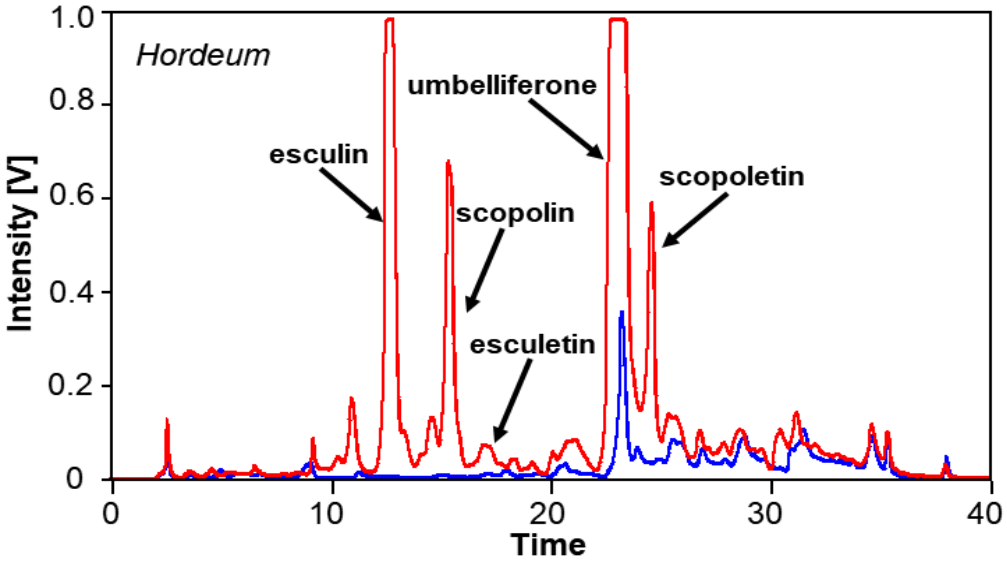

Figure 4-22: Uptake and modification of umbelliferone by excised barley leaves in an upright position. The corresponding HPLC chromatogram of the extract from the control leaves is displayed in blue, this of the treated leaves is given in red.

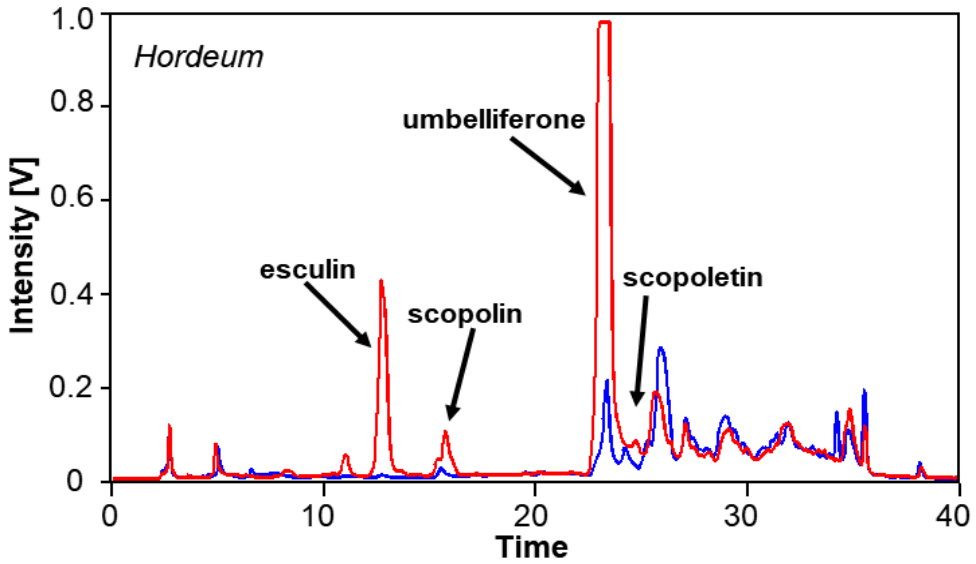

Figure 4-23: Uptake and modification of umbelliferone by excised barley leaves in an upside-down position. The corresponding HPLC chromatogram of the extract from the control leaves is displayed in blue, this of the treated leaves is given in red.

From this, it can be deduced that the absence of glucosidic coumarins in the leaves of the entire seedlings incubated in umbelliferone, cannot be caused and explained by concentration differences due to a xylem translocation. Moreover, since the aglycones are diffusible across the membranes, any strict compartmentation of

coumarins and the glucosyltransferases responsible for the formation of the glucosides could not explain the observed different patterns. Thus, typical spatial effects can also be excluded. Accordingly, the differences in glucosylation must be due to another factor, i.e., differences in the activity of glucosyltransferases involved. As for both approaches, identical plant materials (similar weights of the dry materials) were used, the only explanation for such differences is related to an induction of these enzymes. Corresponding elicitation processes for glucosyltransferases by high concentrations of substances are known from the literature (Tanaka et al., 1990; Pflungmacher et al., 1998) and outlined in detail and argued in the "Discussion" chapter (section 5.3.2).

It has to be mentioned that also in the case of a direct incubation, which putatively induces the relevant glucosyltransferase catalyzing the formation of scopolin and esculin, respectively, no skimmin is formed. Accordingly, as outlined above in Figure 4-11, no glucosylation prior to the hydroxylation or methylation occurs. Nonetheless, the enzyme is glucosylating the putative intermediate esculetin derived from the hydroxylation of umbelliferone to esculin and scopolin (Figure 4-24). Thus, the question arises, why esculetin, or its derivative esculin, respectively is not detectable in the approach, in which umbelliferone is added to the whole seedlings.

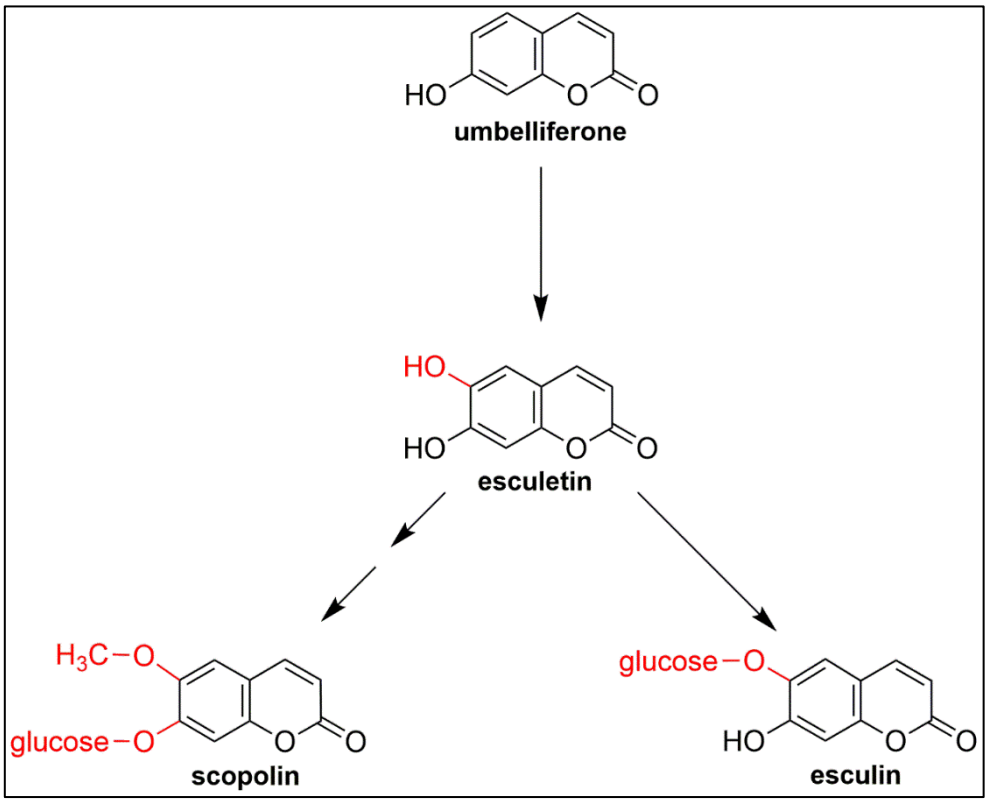

Figure 4-24: Glucosyltransferase is responsible for the attachment of the glucose moiety present in esculin and scopolin, respectively.

4.2.2 Modification of the imported umbelliferone

Two major questions have to be answered, i.e., is skimmin a putative intermediate of scopolin production in barley, and why is no esculetin, or its glucoside esculin, detectable in barley seedlings, whereas large amounts of esculin are present in the cut barley leaves.

4.2.2.1 Involvement of skimmin

As postulated above, the UDP-glucosyltransferase responsible for glucosylation of coumarins has to be induced by the presence of high concentrations of coumarins. As the coumarins can easily diffuse through the biomembranes, the observed differences in the ratios of the various coumarins cannot exclusively be based on differences in their localization but must be – at least in part – due to the substrate specificity of the putative glucosyltransferase. As already mentioned - although

umbelliferone is present in huge amounts- a corresponding glucoside, i.e., skimmin, is not detectable. Consequently, in barley as well as in *Lepidium* this glucoside seems to be not involved in the modification processes. In principle, there might be two possibilities to explain this fact, 1) umbelliferone is glucosylated to yield skimmin, which immediately might be converted by hydroxylation to yield cichoriin, which subsequently is methylated to scopolin. 2) the putatively induced glucosyltransferase is not able to glucosylate umbelliferone.

In the first case, the produced glucoside i.e., skimmin must be directly and efficiently converted to cichoriin, then to scopolin (Figure 4-25).

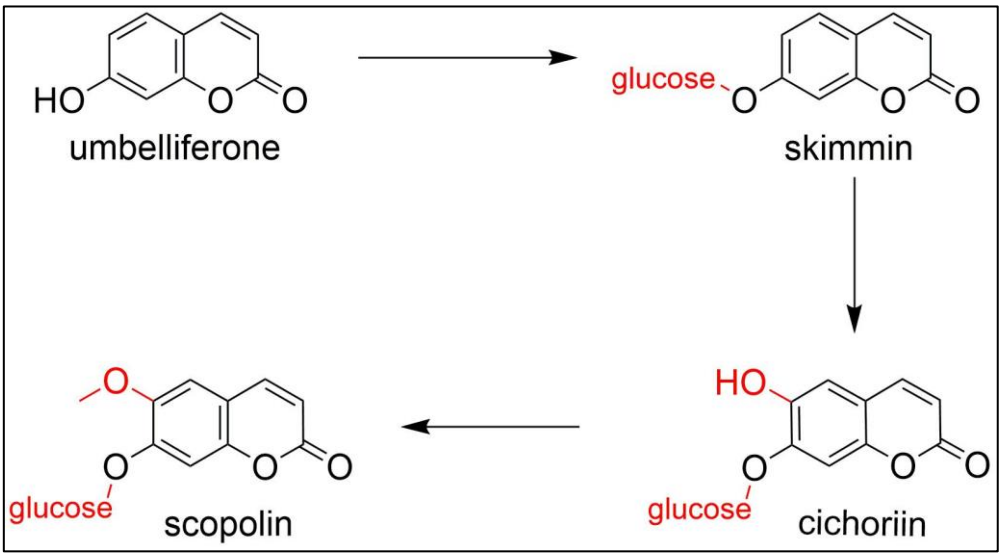

Figure 4-25: "Pathway A", umbelliferone taken up by excised barley leaves is converted to the glucoside scopolin via skimmin as an intermediate.

This, however, would mean that the corresponding hydroxylase is not only accepting skimmin as substrate, but it exhibits such a tremendous high affinity for this glucoside that it cannot be detected at all. Since no cichoriin was detected in the barley leaves, also this intermediate must be efficiently methylated. Accordingly, this option seems to be very unlikely. However, we have to consider that a similar situation also applies for the production of esculin: although high amounts of esculin are present in the barley leaves, only traces of intermediate esculetin are detectable (Figure 4-19; 4-22). Obviously, the corresponding

glucosyltransferase generating esculin reveals such high affinity that only very small amounts of aglycone, i.e., esculetin are detectable (Figure 4-26).

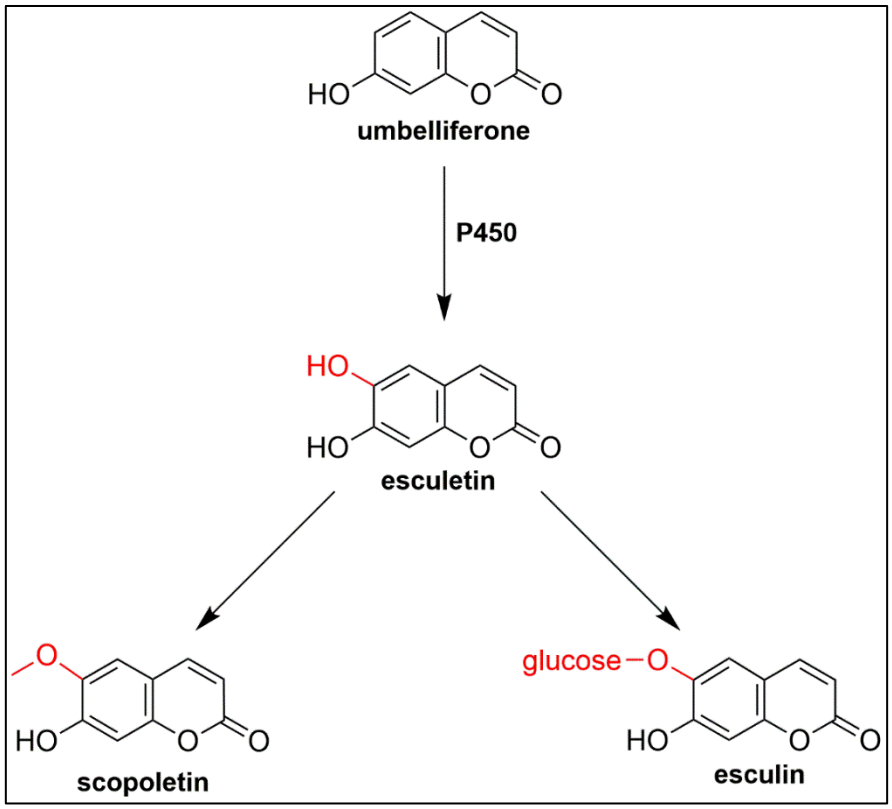

Figure 4-26: "Pathway B", umbelliferone taken up by excised barley leaves is converted to scopoletin and the glucosides esculin and scopolin, where this conversion could occur through the esculetin as an intermediate.

In order to further elucidate the putative pathways, in this study two different approaches had been realized. 1) by application of inhibitors, the conversion of potential intermediate, i.e., skimmin, should be blocked, and 2) by direct application of the alternative intermediate, i.e., esculetin, its concentration was enhanced to increase the generation of the products.

4.2.2.2 Application of P450 enzymes inhibitor

Based on the knowledge of xenobiotics conversion, which are taken up by the plants, most of the various modifications of the foreign substances are catalyzed by cytochrome P450 enzymes (Coleman et al., 1997). A fast and indirect method

could be used to investigate the involvement of such cytochrome P450 enzymes: the application of appropriate enzyme inhibitors or competitive substrates. Naproxen is known to efficiently inhibit many cytochrome P450 enzymes (Abouzeid et al., 2019).

Accordingly, naproxen was applied simultaneously together with umbelliferone to the hydroponic system, in which the barley and garden cress seedlings were grown. After several days, the plants were harvested and analyzed to elucidate the inhibitor effect.

After analyzing the collected samples, a strong reduction of scopoletin generation in the barley seedlings was detected. Obviously, the naproxen was taken up together with umbelliferone, and inhibited its conversion to scopoletin, putatively by inhibiting the enzymes responsible for the corresponding conversion (Figure 4-27; Table 4-2).

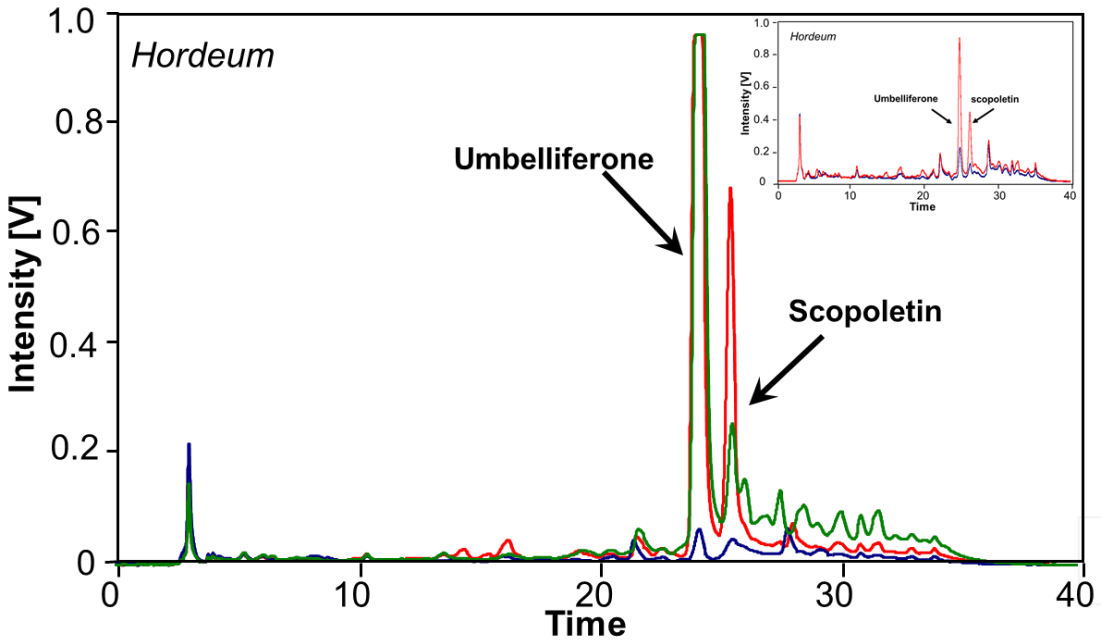

Figure 4-27: Effect of naproxen on the uptake and modification of umbelliferone by barley (*Hordeum vulgare*) seedlings. The corresponding HPLC chromatogram of the extract from the control plants is displayed in blue, this of the treated plants with umbelliferone is given in red, and this of plants treated with both umbelliferone and naproxen is in green. Right upper corner: Figure (4-8) direct application of umbelliferone to barley seedlings.

Due to the presence of several other fluorescing compounds, the chromatograms of extracts from *Lepidium,* are quite complex. However, when concentrating on the coumarins, various aspects relevant to the coumarin modification became obvious. As already outlined (Figure 4-10), the overall concentration of endogenous scopoletin is not affected by the umbelliferone uptake. Also as in the first row of experiments, the concentration of umbelliferone is enhanced. The application of naproxen does not change these outcomes. However, naproxen leads to a significant decrease in esculin and scopolin production(Figure 4-28; Table 4-2).

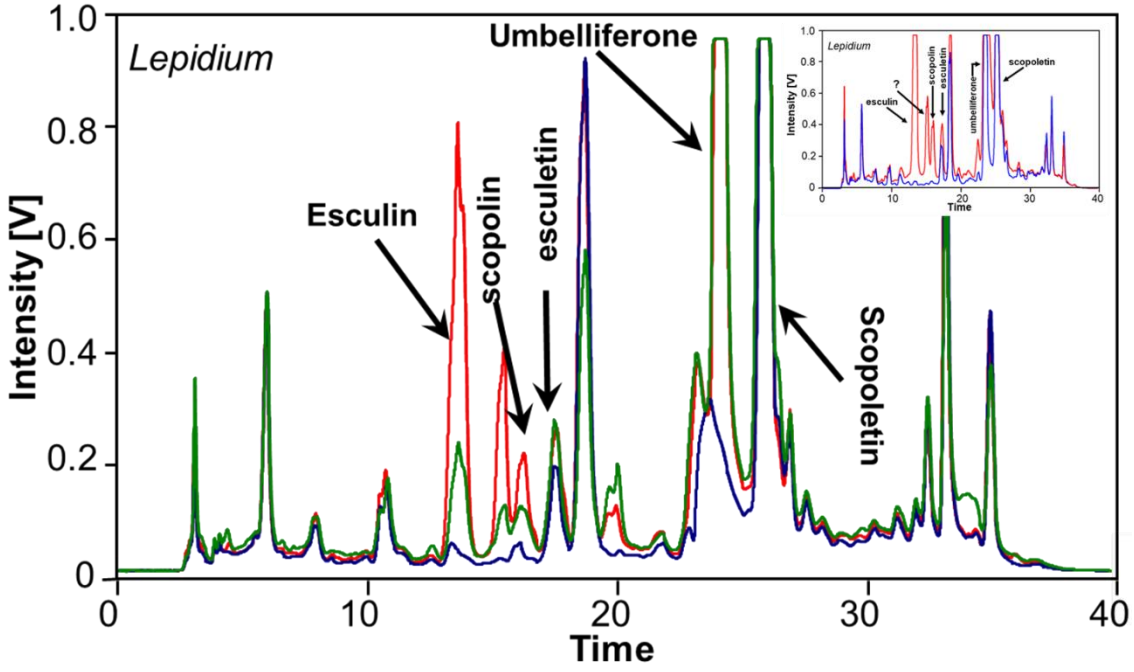

Figure 4-28: Effect of naproxen on the uptake and modification of umbelliferone by garden cress (*Lepidium sativum*). The corresponding HPLC chromatogram of the extract from the control plants is displayed in blue, this of the treated plants with umbelliferone given in red, and the green for plants treated with both umbelliferone and naproxen. Right upper corner: Figure (4-10) direct application of umbelliferone to garden cress seedlings.

As postulated, both compounds share the same intermediate, i.e., esculetin. This is underlined by the fact, that its production declines in the plants treated with naproxen due to the putative inhibition of the cytochrome P450 enzyme responsible for the hydroxylation of umbelliferone (Figure 4-29).

As consequence, the extent of esculetin glucosylation decreases. In contrast to these clear coherences, the naproxen-related decrease of scopolin production cannot be explained easily at this point. Although all plants reveal tremendous high concentrations of scopoletin, only in plants treated with umbelliferone, the scopoletin-glucoside is detectable. Obviously, scopolin is exclusively generated from scopoletin derived from the imported umbelliferone. This is fully in accordance with the finding that scopolin production decreases when the concentration of esculetin is declined by the addition of naproxen. (Table 4-2).

To summarize the results elaborated and to expound the effect of naproxen, the concentrations of the umbelliferone and its derivatives in barley and garden cress are compiled in Table 4-2.

Table 4-2: Concentration of umbelliferone and its derivatives in the acceptor barley and garden cress plants, after treating them with umbelliferone and naproxen.

	Acceptor plants	Concentration (µg/g d.w.)				
		umbelliferone	scopoletin	scopolin	esculetin	esculin
H. vulgare	*control*	0.2 ± 0.2	0.8 ± 0.1	-	-	-
	+ umbelliferone	62.7 ± 4.1	8.2 ± 1.3	-	-	-
	+ umbelliferone + Naproxen	65.2 ± 9.4	2.5 ± 1.4	-	-	-
L. sativum	control	22.1 ± 0.9	39.5 ± 1.2	1.2 ± 0.5	350.2 ± 26.9	0.2 ± 0.1
	+ umbelliferone	41.6 ± 2.6	49.0 ± 2.1	6.2 ± 0.6	553.9 ± 30.5	18.3 ± 6.2
	+ umbelliferone + Naproxen	46.0 ± 2.3	48.9 ± 3.1	4.8 ± 0.4	495.9 ± 20.6	7.9 ± 0.9

The concentrations of the various coumarins have been quantified by HPLC. The concentrations represent the mean values of three independent experiments applying about 15 to 20 seedlings for each approach. d.w: dry weight

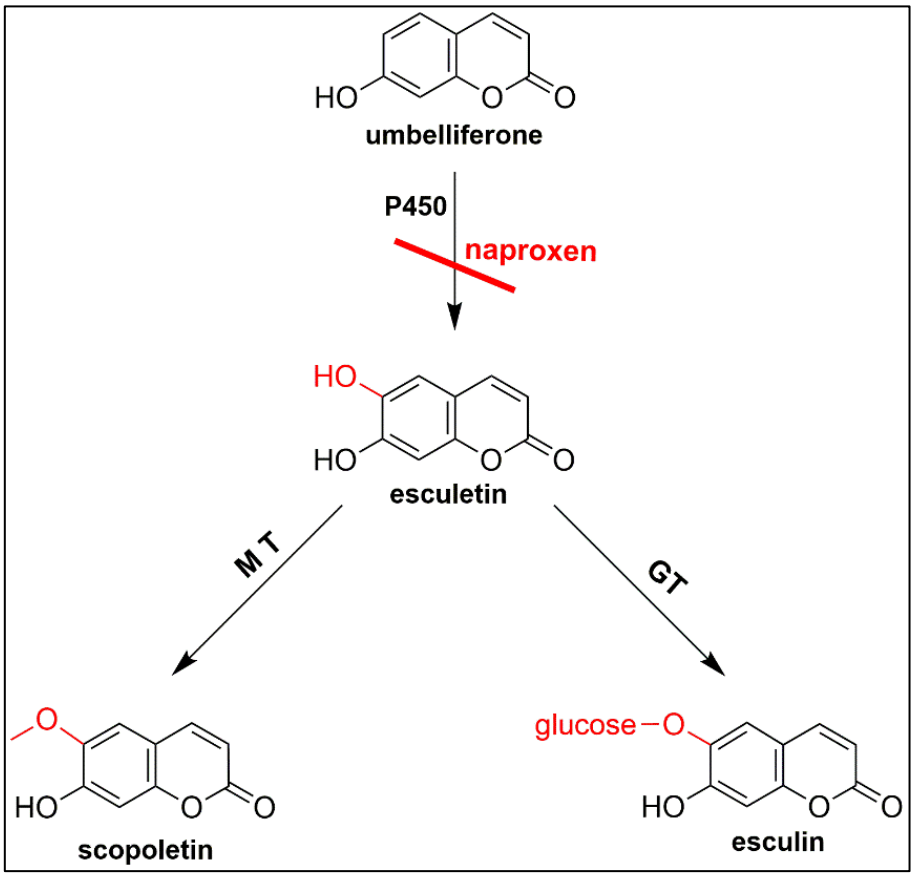

Figure 4-29: Addition of naproxen to the garden cress (*Lepidium sativum*) together with umbelliferone caused a strong reduction in esculin production, due to the inhibition of P450 enzymes which generate esculetin by hydroxylation. Glucosyltransferases are constitutively expressed.

In this context, we have to consider that – in principle – scopoletin is able to pass biomembranes. Thus, no spatial compartmentation can be responsible for the difference in the fate of genuine scopoletin, already present in the control plants, and that derived from esculetin generated by the hydroxylation of umbelliferone. This issue will be outlined in detail in the "Discussion" chapter.

When evaluating these results with respect to the various options of the umbelliferone conversions, we have to consider two issues.

1) the differences in the ratio of umbelliferone to scopoletin in leaves of barley (Figure 4-8) in relation to the guttation droplets (Figure 4-14), which points to the fact that a share of umbelliferone might already be modified in the roots.

2) the massive differences in the amount of coumarin glucosides between the entire seedlings and the cut leaves (Figure 4-8; 4-19, respectively). To pay regard to these coherences and to further elucidate the complex situation, the P450 enzyme inhibitor also applied simultaneously with umbelliferone to cut barley leaves. After five days, the leaves were washed to remove the excess umbelliferone putatively sticking to the leaf surface and analyzed.

Addition of the naproxen simultaneously with the umbelliferone to the cut leaves caused a strong reduction in the amount of the produced scopolin, scopoletin compared to the leaves incubated only with umbelliferone, which showed a massive production in the scopoletin, scopolin, and esculin (Figure 4-30). However, in the cut leaves, naproxen did not significantly influence the generation of esculin. Since naproxen reduces the conversion of umbelliferone to scopoletin and scopolin but not to esculin (Figure 4-30), it can be deduced that naproxen – in contrast to the situation in *Lepidium* - does not inhibit the activity of P450 enzymes responsible for the hydroxylation of umbelliferone to yield esculetin, which subsequently is glucosylated to esculin.

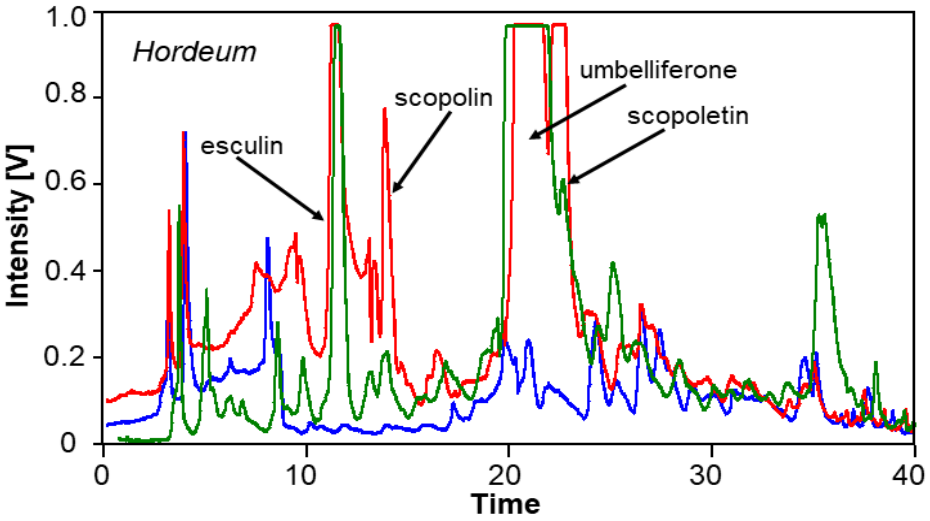

Figure 4-30: Effect of naproxen on the uptake and modification of umbelliferone by excised leaves (upright position). The corresponding HPLC chromatogram of the extract from the control leaves is displayed in blue, this of the treated leaves with only umbelliferone is given in red, and the green for plants treated with both umbelliferone and naproxen.

Acutally, naproxen reduces the generation of scopoletin, putatively by inhibiting the related methyltransferase (Figure 4-31). Corresponding inhibitory effects of naproxen on methyltransferases are well known (Oselin and Anier, 2007). Since in any case, skimmin accumulation was not detected, the proposed "pathway A" (Figure 4-25) could be excluded, and pathway "B" (Figure 4-26) seems to be likely.

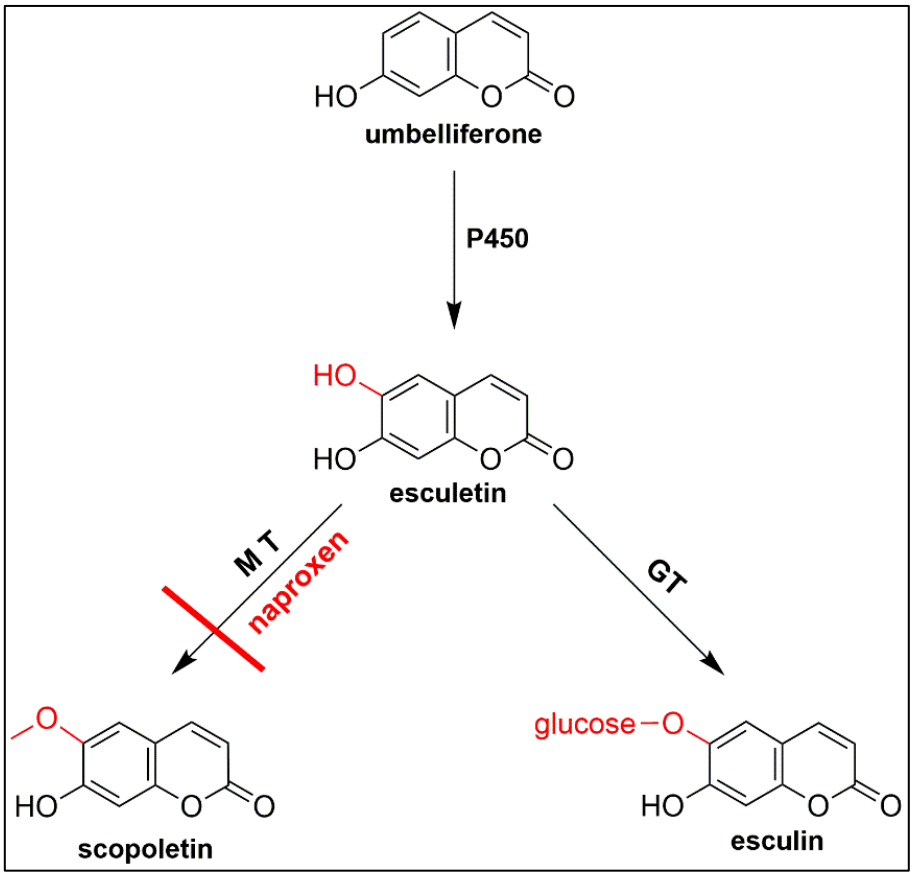

Figure 4-31: The addition of naproxen to the barley (*Hordeum vulgare*) together with umbelliferone caused a strong reduction in scopoletin production, due to the inhibition of methyltransferase enzyme (MT). Glucosyltransferases activated after an induction.

4.2.2.3 Application of esculetin to cut barley leaves

In a further approach to elucidate the conversion of umbelliferone to esculin, scopoletin, and scopolin, respectively, esculetin as a putative intermediate was applied exogenously to the excised barley leaves.

After 5 days of incubating the cut barley leaves with esculetin, several compounds were massively generated (Figure 4-32). The comparison of their retention times

with those of various coumarins reveals that these substances correspond to scopoletin, scopolin, and esculin as shown in Figure 4-32 below.

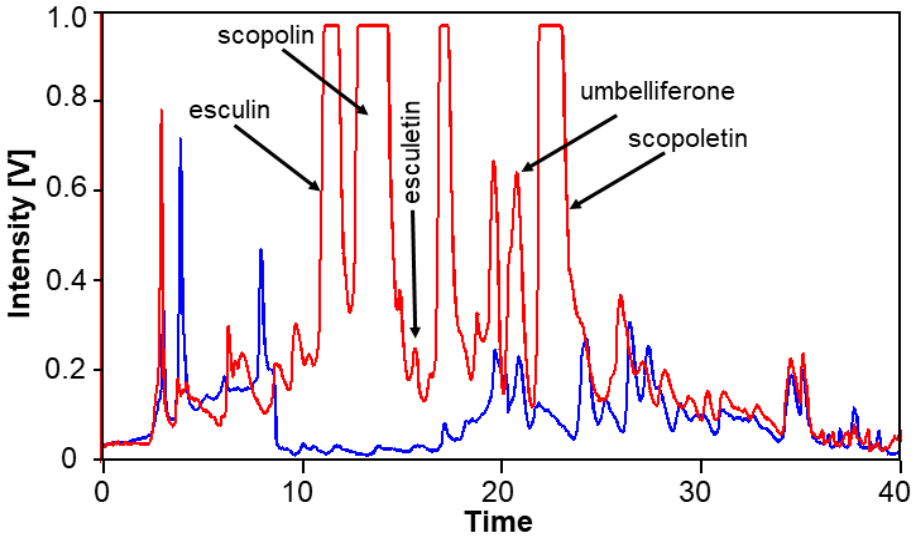

Figure 4-32: Modification of esculetin by excised barley (*Hordeum vulgare*) leaves (upright position). The corresponding HPLC chromatogram of the extract from the control leaves is displayed in blue, this of the treated leaves with esculetin is given in red.

The enhancement of the intermediate concentration, i.e., esculetin strongly forces the modification reactions in the direction of the production of several coumarinic compounds, i.e., esculin, scopolin, and scopoletin. In this context, the production of the modified products from umbelliferone is more likely to occur by firstly hydroxylating it to esculetin, before methylating and/or glucosylating the intermediates to produce the different coumarins. As a result, their production is proceeding via the esculetin pathway (Figure 4-26) rather than the other pathway where the skimmin is the intermediate (Figure 4-25).

Interim conclusion

According to the Green liver concept, the compounds are detoxified according to a certain general system involving oxidation, hydroxylation, and conjugation steps. As shown above, one compound i.e., umbelliferone behaved and metabolized differentially in the various plant species, where in some species it was just accumulated to a high extent, in others, it was accumulated and metabolized.

However, the derivatized compounds were different according to the plant species. In certain species, umbelliferone is hydroxylated and glucosylated, while in the other it was further methylated and glucosylated. Such differences are related to the enzymatic content of the different species, in which the foreign compound is randomly incorporated and modified. Accordingly, these results do not agree with the general detoxification system proposed by the green liver concept and thus, point to the fact that the so-called green liver concept seems to be inappropriate!

4.3 Uptake and modification of esculetin

In *Linum*, *Pisum*, and *Raphanus* the imported umbelliferone is just accumulated without any modification. Obviously, the related P450 enzymes in these species are not able to modify this coumarin. Yet, to elaborate further information on putative subsequent biotransformations, i.e., methylation and or glucosylation, esculetin - known to be the intermediate in barley and *Lepidium* - was added to the culture medium, in which the seedlings of the various species (*Lepidium*, *Hordeum*, *Pisum*, *Raphanus,* and *Linum*) were grown. It is worthy to note again that the esculetin is relatively unstable and oxidized when added to the culture medium (Appendix, Figure A-1). In consequence, in the course of the experiment, the roots of the seedlings, as well as the entire media, turned black. After 5 days of incubating the esculetin with the previously mentioned seedlings, the aerial parts of the tested seedlings were collected and analyzed.

Unlike in the case of umbelliferone application, no massive esculetin accumulation could be detected. As the log*P*-values of umbelliferone and esculetin are similar and thus their membrane permeability, the lack of coumarin accumulation should be due to the observed blackening reactions. Nonetheless, the quantity and the composition of the imported coumarins and their putative derivatives strongly differs between the seedlings of the various plants.

In *Pisum* and *Linum*, the chromatograms of the control and the treated seedlings show no difference, as shown in Figure 4-33 and Figure 4-34, respectively. In both

plants, neither esculetin nor any other modified products were detected. Obviously, the blackening reactions completely have suppressed the uptake of esculetin.

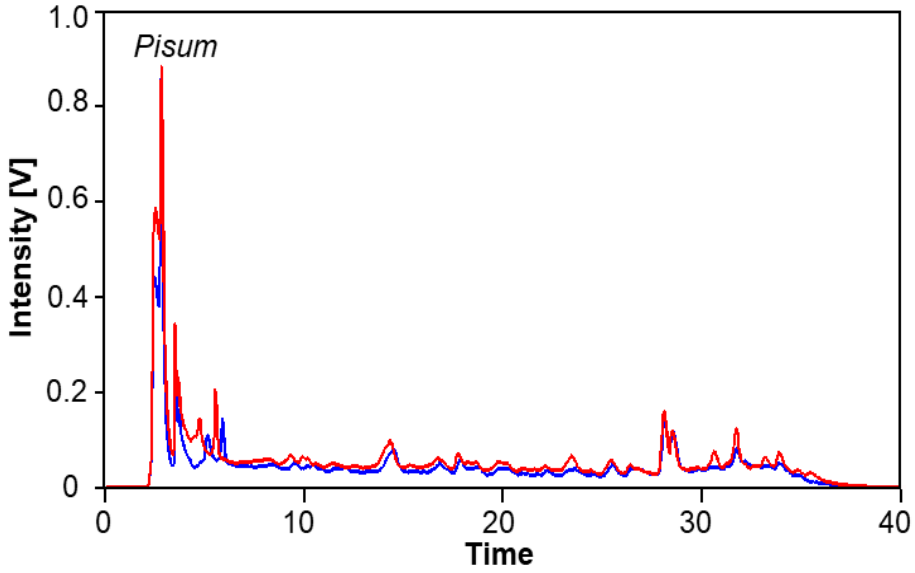

Figure 4-33: Uptake and modification of esculetin by pea seedlings (*Pisum sativum*). The corresponding HPLC chromatogram of the extract from the control plants is displayed in blue, this of the treated plants is given in red.

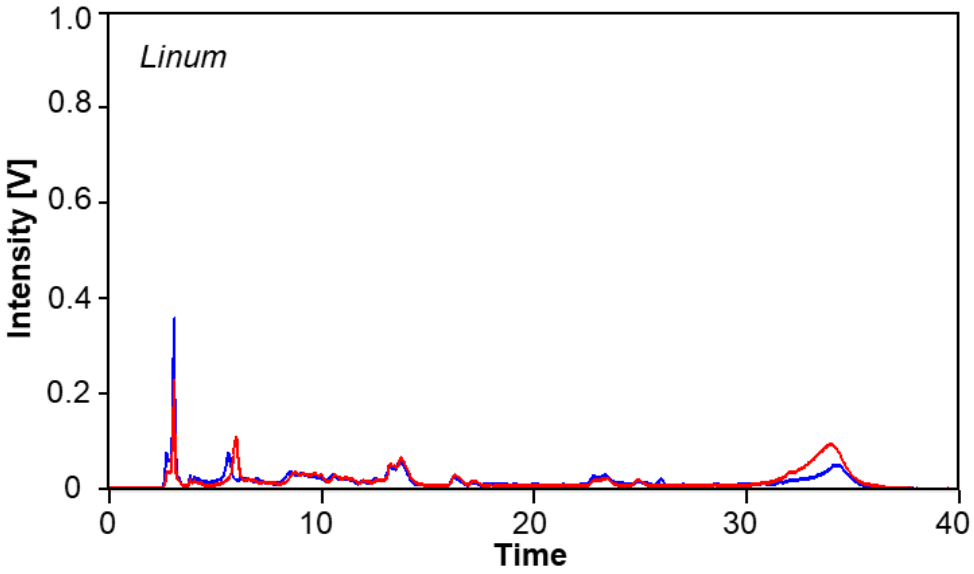

Figure 4-34: Uptake and modification of esculetin by flax seedlings (*Linum usitatissimum*). The corresponding HPLC chromatogram of the extract from the control plants is displayed in blue, this of the treated plants is given in red.

In the other three plant species employed, i.e., *Lepidium*, *Hordeum*, and *Raphanus*, the situation turned out to be different. Indeed, also in the seedlings of these

species, no changes in the esculetin concentration could be observed: in barley no esculetin was detected at all (Figure 4-35), and in *Lepidium* and *Raphanus* the esculetin concentration was the same in the controls as well as in the treated seedlings (Figure 4-36; 4-37), respectively. However, in all of these three species, the treated seedlings contained a significantly higher concentration of other coumarins, putatively generated from the imported esculetin.

In the barley seedlings treated with esculetin, high amounts of scopoletin are present (Figure 4-35).

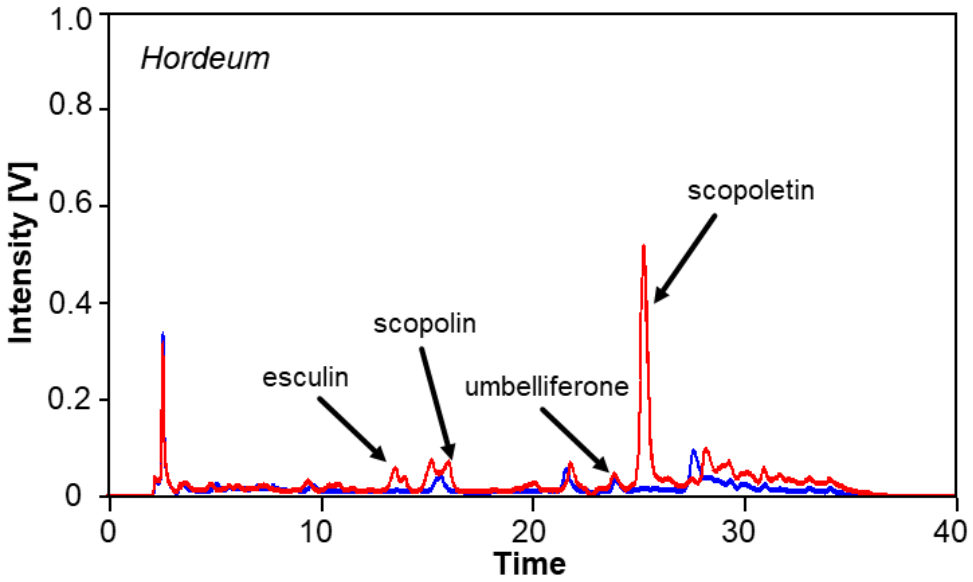

Figure 4-35: Uptake and modification of esculetin by barley (*Hordeum vulgare*). The corresponding HPLC chromatogram of the extract from the control plants is displayed in blue, this of the treated plants is given in red.

As no scopoletin is present in the medium (Appendix, Figure A-4), esculetin must have been taken up and converted to scopoletin. Interestingly, despite the very high concentration of the putative product scopoletin, no accumulated esculetin could be detected. Obviously, the affinity of the methyltransferase towards esculetin must be high that the imported esculetin is exhaustively converted into scopoletin. However, a small share of esculetin is glucosylated to esculin. Accordingly, the methyltransferase successfully competes with the glucosyltransferase, which putatively exhibits a higher affinity towards esculetin.

In this context, also the generation of scopolin has to be mentioned. Although the concentration of scopoletin is remarkably high, the concentrations of esculin and scopolin are nearly the same. In principle, there are two explanations for this finding: either the glucosyltransferase has a tremendously higher affinity towards esculetin than towards scopoletin, or, parts of esculin are converted to scopolin.

In summary: esculetin is imported by the barley roots and efficiently methylated to scopoletin. Yet, a small share of the imported coumarin is converted to esculin and scopolin, respectively.

In the case of garden cress, the concentration of the scopoletin -the main coumarin- didn't enhance in the treated seedlings after esculetin feeding (Figure 4-36). On the other hand, another substance was enhanced after taking up the esculetin, this compound is identified by HPLC as esculin.

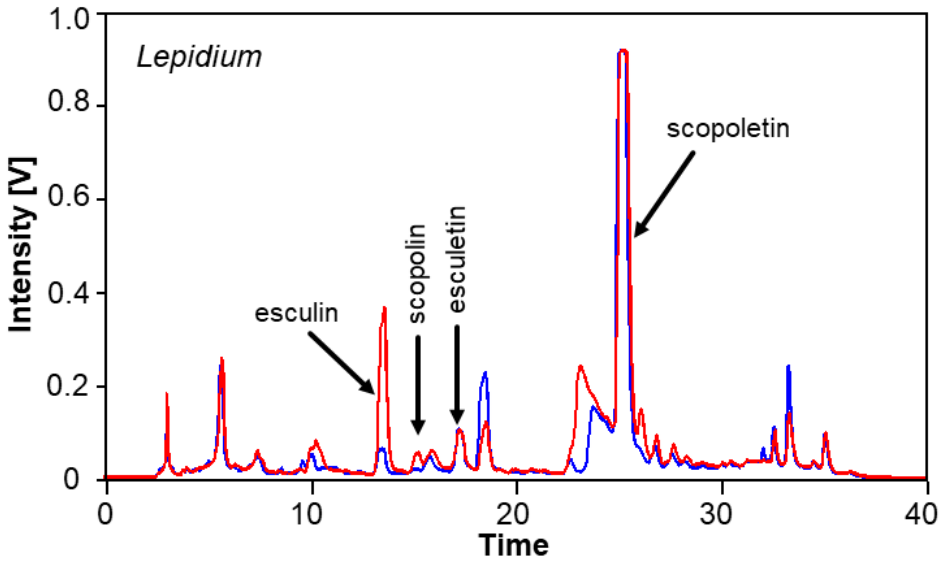

Figure 4-36: Uptake and modification of esculetin by garden cress (*Lepidium sativum*). The corresponding HPLC chromatogram of the extract from the control plants is displayed in blue, this of the treated plants is given in red.

Despite the high concentration of the genuine scopoletin, no glucoside i.e., scopolin was produced in the control plants. On the other side, the addition of esculetin slightly induced the production of scopoletin which seems to be converted directly into its glucoside i.e., scopolin.

Also in *Raphanus*, the concentration of the genuine scopoletin had not been affected by the exogenous application of esculetin (Figure 4-37). However, unlike the other studied plant species, the esculetin was taken up efficiently to a very large extent, then the imported esculetin is directly glucosylated to esculin and accumulated in the aerial parts as a glucoside. It seems that the conversion of esculetin to esculin creates a pulling force to import more esculetin through roots into the leaves, to be glucosylated into esculin. Additionally, a small share of the imported esculetin is also modified by methylation, and subsequently by glucosylation to yield scopolin the glycosidic form of scopoletin as shown in Figure 4-37.

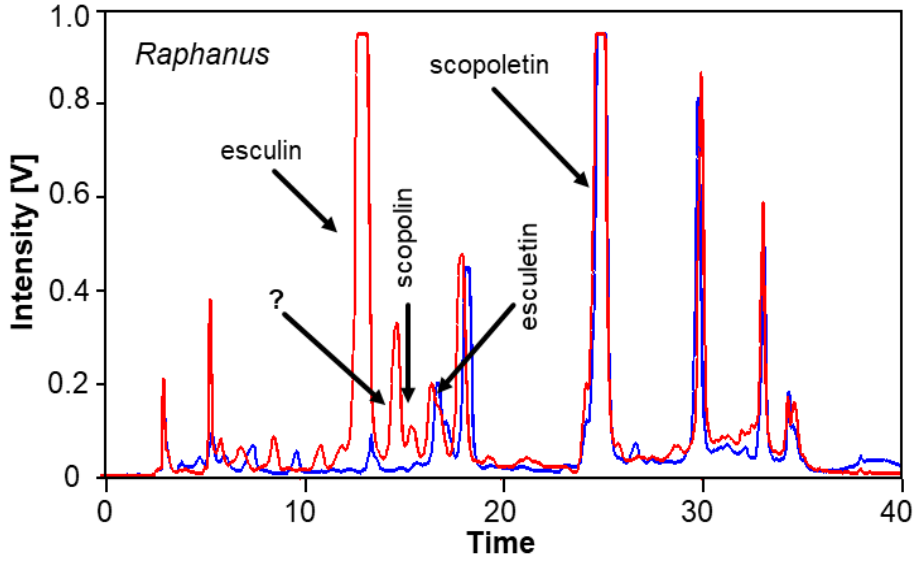

Figure 4-37: Uptake and modification of esculetin by radish seedlings (*Raphanus sativus*). The corresponding HPLC chromatogram of the extract from the control plants is displayed in blue, this of the treated plants is given in red.

It is worthy to mention that the *Raphanus* seedlings contain scopoletin genuinely, but no scopolin was produced, on the other hand, adding esculetin externally to the seedlings induces the enzymes that activate different reactions to give the glycosidic form of scopoletin i.e., scopolin. Therefore, the same question arises: why the endogenous compound is not converted by the plant's enzymes while adding the same compound externally induces them to biotransform it into different derivatives. Moreover, another question may arise, why the rate of the

esculetin import is higher in *Raphanus* than the other species, since the available un-oxidized esculetin is limited, due to its fast oxidation.

In summary, the esculetin was taken up by the seedlings of *Lepidium*, *Hordeum*, and *Raphanus* through their roots. This is in accordance with the log*P* of esculetin that confers it the ability to pass biomembranes. In contrast, in flax and pea, this uptake is suppressed. Subsequently to its uptake, the imported esculetin was modified to yield various compounds (Figure 4-38). These modified coumarins had been identified according to their retention times and their fluorescence spectra in comparison with authentic standards.

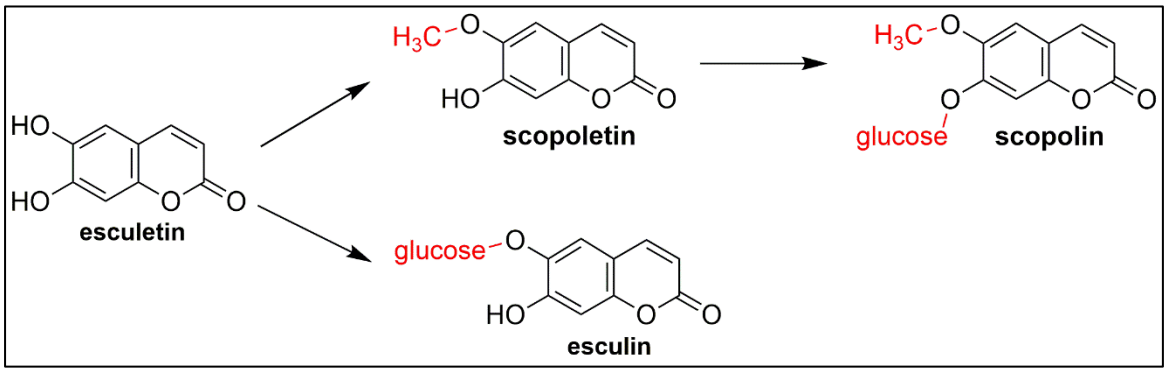

Figure 4-38: Different derivatives were produced from esculetin after its uptake by the studied plant species i.e., barley (*Hordeum vulgare*), garden cress (*Lepidium sativum*), and radish (*Raphanus sativus*).

According to these results, one issue is of special interest and requires an explanation: what is the reason for the lacking uptake of esculetin in flax and pea seedlings. One obvious explanation might be related to a putative larger extent of oxidative processes, may be caused by differences in the redox potential of the rhizosphere. Indeed, by employing high concentrations of reducing agents, the oxidation of esculetin could be prevented – but in this case, the roots would die back due to lack of oxygen. The entire issue related to the strongly reduced availability of esculetin is outlined in detail in the "Discussion" chapter (section 5.2.2).

An alternative explanation for the tremendous high uptake of esculetin in *Rhaphanus* (displayed by the massive accumulation of esculin, Figure 4-37) might be due to the fact that esculetin is instantly glucosylated to esculin, which results in a permanent high diffusion gradient of esculetin between rhizosphere and root cells. In the same manner, the slight uptake of esculetin in *Lepidium* might be explained; obviously, in these seedlings the glucosylation to yield esculin is less effective, resulting in a lesser diffusion gradient of esculetin between rhizosphere and root cells. In analogy, in barley, the diffusion gradient is generated by methylation of the imported esculetin to generate scopoletin. This - in conclusion – would mean that the extent of esculetin import depends on the capacity of its derivatization. However, since such coherences are not valid for the uptake of umbelliferone, a further factor must be involved. In this context, it has to be considered that the concentration of esculetin in the medium is strongly decreased due to its oxidation, whereas the umbelliferone concentration is maintained at a very high level. Accordingly, the diffusion gradient between rhizosphere and root cells is far higher in the case of umbelliferone than that of esculetin, resulting in massive differences in the uptake of umbelliferone and esculetin. There are only two possibilities to enhance the concentration gradient, i.e., to decrease the internal concentration or to enhance the exogenous concentration. Indeed, the internal concentration of the imported esculetin could be strongly and permanently reduced, by its effective modification. As a result, as mentioned above, a significant uptake will occur. For increasing the exogenous concentration of esculetin, continuously new esculetin should be added in order to supplement the coumarin, which is lost by its oxidation. Alternatively, the application of esculetin could be performed by adding continuously low amounts. A suitable method for this could be the application of its glucoside esculin, from which the aglycone is liberated by the action of β-glucosidases present in the apoplastic space of the root cells.

4.4 Uptake and modification of esculin

As mentioned above, one approach to keep a continuous supply of "non-oxidized esculetin" to the seedlings is adding its glucosidic form to the medium, which should be cleaved by the glucosidases, liberating the aglycone. The postulated cleavage of coumarin-glucosides was confirmed by employing the non-fluorescing 4-methylumbelliferyl-glucoside. When hydrolyzed, the strong fluorescent 4-methylumbelliferone is generated, which can be easily recognized. In a corresponding approach with barley seedlings, already after few minutes, the blue fluorescence of methylumbelliferone appeared (Figure 4-39), documenting that β-glucosidases in the medium indeed are able to cleave coumarin-glucosides.

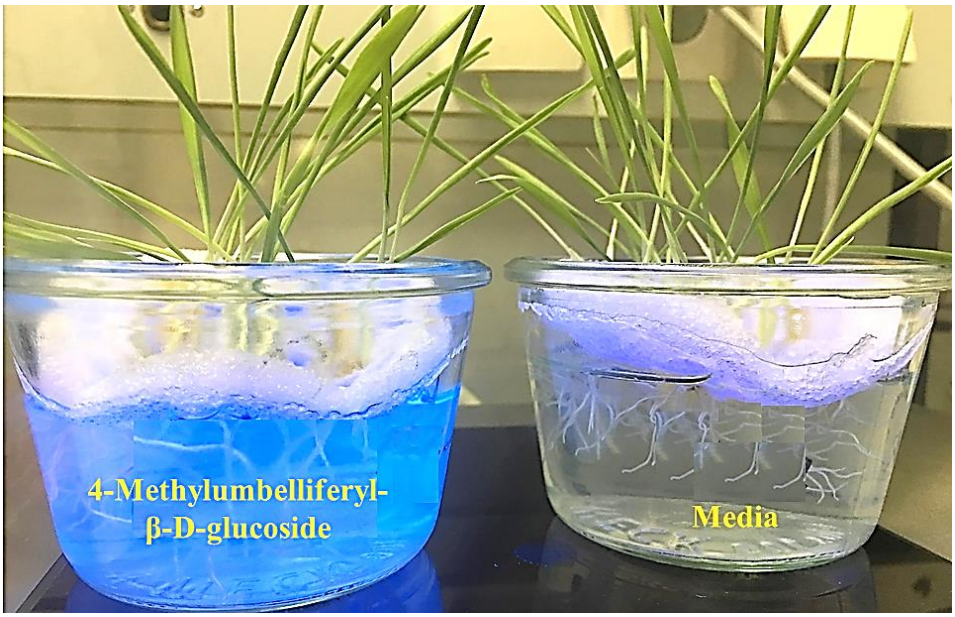

Figure 4-39: The fluorescence of 4-Methylumbelliferyl-β-D-glucoside after its cleavage and removal of the sugar moiety by glucosidases in the growth media.

Moreover, the liberation of esculetin from the esculin added to the media was verified by HPLC analyses (Appendix, Figure A-5). In the media of all employed species, the hydrolysis of the added esculin was verified.

Five days after the addition of esculin, the seedlings were harvested and analyzed. According to the HPLC analyses, no coumarins were taken up and accumulated in the seedling of *Pisum* (Figure 4-40) and *Linum* (Figure 4-41).

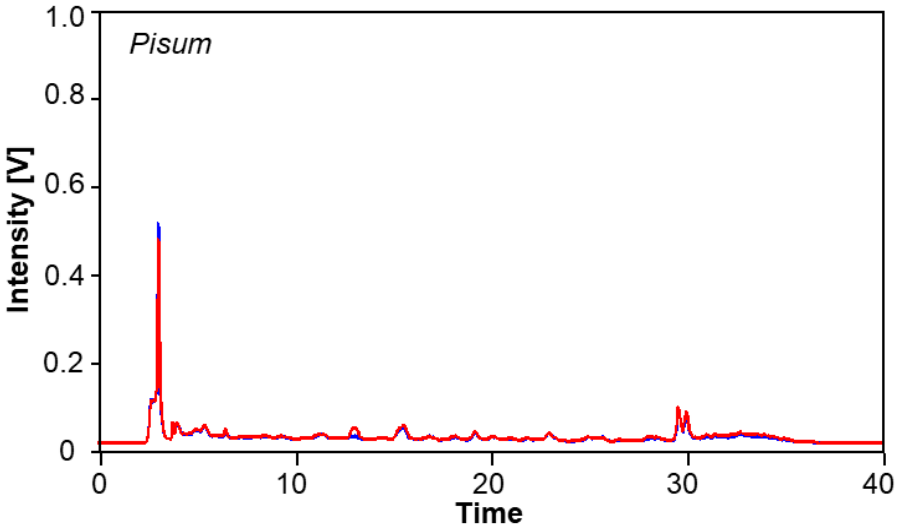

Figure 4-40: Uptake and modification of esculin by pea seedlings (*Pisum sativum*). The corresponding HPLC chromatogram of the extract from the control plants is displayed in blue, this of the treated plants is given in red.

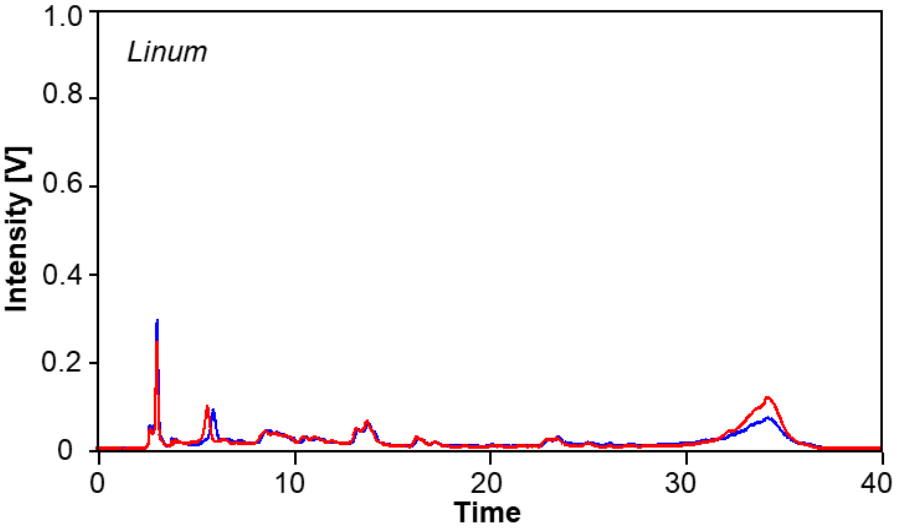

Figure 4-41: Uptake and modification of esculin by flax seedlings (*Linum usitatissimum*). The corresponding HPLC chromatogram of the extract from the control plants is displayed in blue, this of the treated plants is given in red.

This is fully in accordance with the outcome of the direct application of esculetin to *Pisum* and *Linum* seedlings (Figure 4-33; Figure 4-34), respectively.

Also, in this approach, no other coumarinic compounds could be detected, which may result from the modification or the biotransformation of esculin in the mentioned species. In addition, this experiment exhibits that in the seedlings of these both species no transporters are present in the roots, which are able to catalyze the uptake of coumarin-glucosides.

In the case of adding esculin to seedlings of *Lepidium*, the plant that already contains high amounts of scopoletin, the content of this coumarin is strongly enhanced (Figure 4-42), an issue which was not realized in the experiments of direct application of esculetin (Figure 4-36), putatively due to the very high endogenous contents of scopoletin. Moreover, in analogy to the direct application of esculetin, also the content of esculin and scopolin are significantly enhanced when esculin is added to the medium.

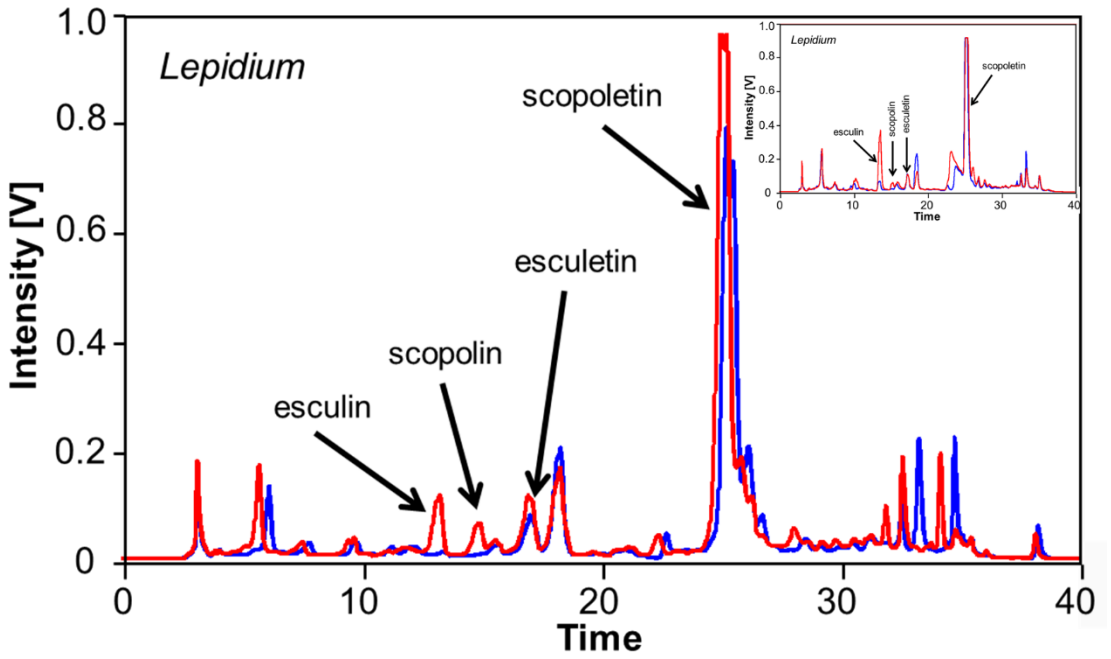

Figure 4-42: Uptake and modification of esculin by garden cress (*Lepidium sativum*). The corresponding HPLC chromatogram of the extract from the control plants is displayed in blue, this of the treated plants is given in red. Right upper corner: Figure (4-36) direct application of esculetin to garden cress seedlings.

This contradictory situation is discussed in detail in the "Discussion" chapter (sections 5.3.1; 5.3.2). In analogy to *Pisum* and *Linum*, also the experiment employing seedlings of *Lepidium* exhibits that no transporters are present, which are able to catalyze the uptake of coumarin-glucosides

In barley, the application of esculin exhibited the same results as in the case of direct application of esculetin, i.e., a large generation of scopoletin and minor amounts of esculin, whereas the putative intermediate esculetin could not be detected; putatively due to its effective transformation (see above) to yield esculin and scopoletin, by glucosylation or methylation, respectively (Figure 4-43).

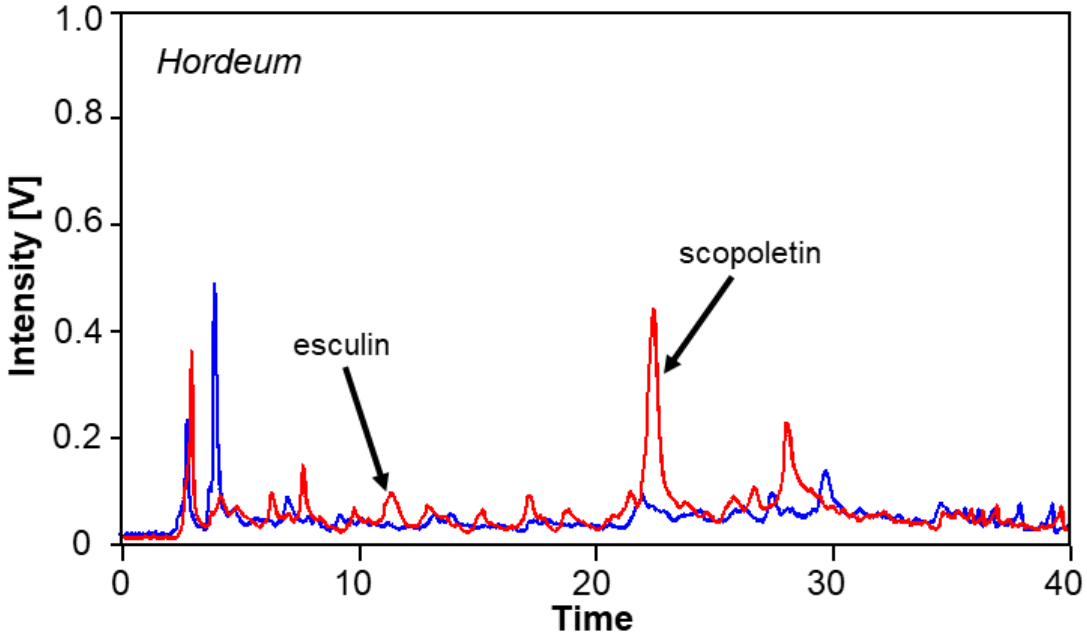

Figure 4-43: Uptake and modification of esculin by barley (*Hordeum vulgare*). The corresponding HPLC chromatogram of the extract from the control plants is displayed in blue, this of the treated plants is given in red.

Overall, this experiment verifies that esculetin derived from the hydrolysis of esculin is effectively taken by the roots and modified to scopoletin. However, due to the enhanced occurrence of esculin - at least to a minor extent - the involvement of a transporter capable to catalyze the import of esculin could not be fully excluded.

In the case of *Raphanus*, the main difference between control and treated plants is due to the very high accumulation of esculin (Figure 4-44). The same behavior was also documented in the case of a direct application of esculetin (Figure 4-37). Obviously, esculetin - either applied directly or as a product of esculin hydrolysis - is taken up efficiently by the *Raphanus* seedlings and converted to esculin. Nonetheless, as already mentioned for barley, it can't be excluded that at least minor amounts of esculin might be taken up via a transporter.

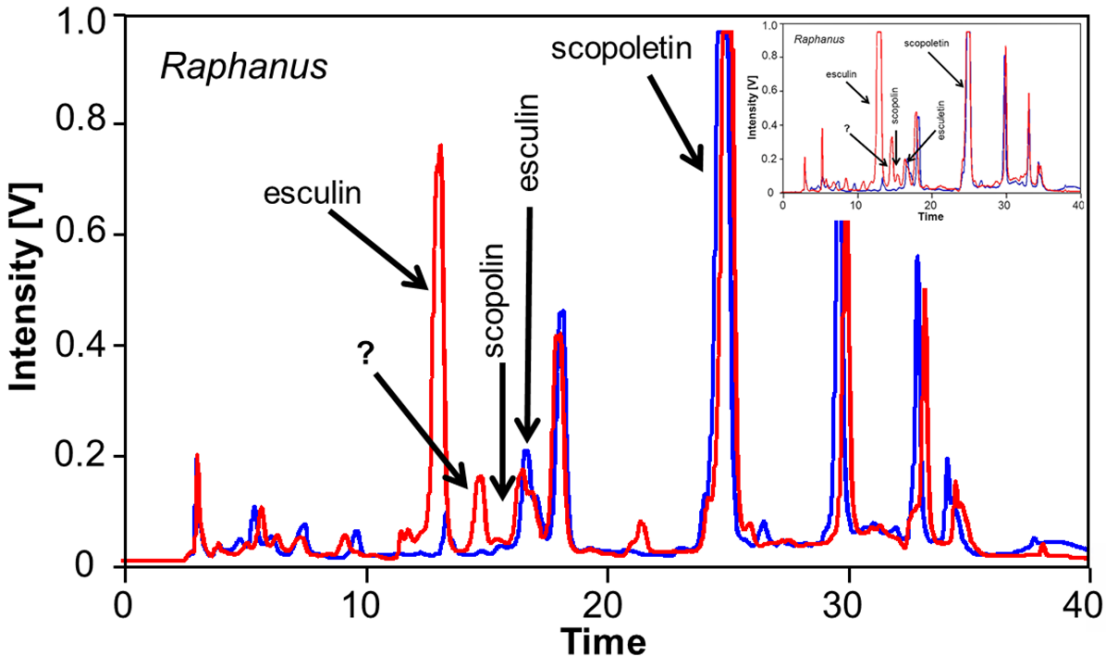

Figure 4-44: Uptake and modification of esculin by radish seedlings (*Raphanus sativus*). The corresponding HPLC chromatogram of the extract from the control plants is displayed in blue, this of the treated plants is given in red. Right upper corner: Figure (4-37) direct application of umbelliferone to radish seedlings.

The approach to apply esculin to the media was aimed to increase or maintain the exogenous concentration of esculetin, which declines rapidly due to its oxidation. The hydrolysis by apoplastic ß-glucosidases should ensure a continuous supply. Unfortunately, this intention did not solve the problem of the ongoing oxidation, and accordingly quite the same results had been achieved. Nonetheless, it can be concluded that esculetin - in accordance with its log*P* - is taken up by the seedlings of *Lepidium*, *Hordeum*, and *Raphanus* through their roots, whereas this coumarin is

not imported by flax and pea seedlings. This finding is in contradiction to the import of umbelliferone, which is taken up by all seedlings. As mentioned above, the only explanation for this different behavior is due to differences in the concentration gradient between rhizosphere and root cells. Whereas the internal concentration of imported coumarins strongly depends on the various modification processes, the external concentration is massively influenced by oxidation processes. Unfortunately, it turned out that any approach to bypass this option by a continuous supply of esculetin was not successful. Accordingly, the underlying coherences have to be clarified theoretically (see "Discussion" chapter, section 5.2.2).

In the context of coumarin uptake, another possibility has to be considered, i.e., the direct uptake of esculin. Yet, since the hydrophilicity of esculin – due to its glucose moiety - is relatively high (its log*P* is lower than -1) it could not pass freely the biomembranes. Accordingly, a question arises, whether a transporter might be involved in a putative uptake of esculin, resulting in concurrent uptake of esculin (via a transporter) and esculetin generated by the hydrolysis of its glucoside. In order to differentiate between these options, the feeding of esculin to radish and barley seedling had been performed in the presence of β-glucosidase inhibitors that prevent the hydrolytic cleavage of the applied esculin.

4.4.1 Application of Glucosidase inhibitors

As outlined, apart from the attempt to maintain the availability of esculetin for a longer period, the application of esculin might also be suitable to elucidate whether or not putative transporters might be involved in the uptake of esculin, as it is reported for various xenobiotics, which are not able to pass freely through biomembranes (Benadiba and Maor, 2016). According to the coherences mentioned above, a mixture of N-glucosyl- and N-Galactosyl-piperidine was added as potent glucosidase inhibitors (De Melo, 2006; Wardrop and

Waidyarachchi, 2010) to the culture medium before adding esculin. After five days, the seedlings were harvested and analyzed.

As exposed already in the previous experiments, the barley seedlings treated solely with esculin, exhibited large amounts of scopoletin. In contrast, the seedlings which were grown in media, in which the hydrolysis of esculin was blocked by the addition of glucosidase inhibitors (Appendix, Figure A-5), no scopoletin was present (Figure 4-45).

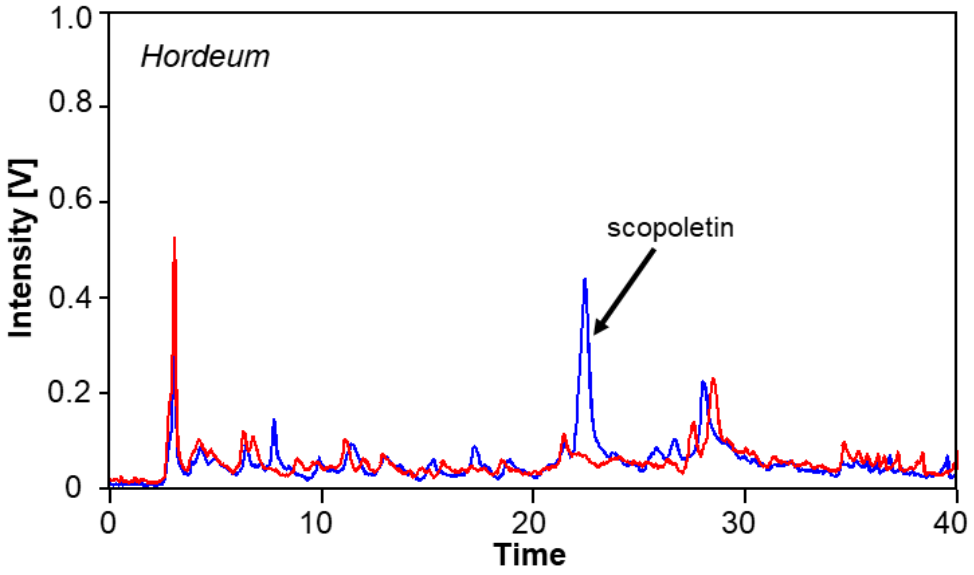

Figure 4-45: Hydrolysis of esculin by glucosidases in barley (*Hordeum vulgare*). Esculin was applied after the application of glucosidase inhibitors into the media. The corresponding HPLC chromatogram of the extract from plants incubated with only esculin is displayed in blue, this of plants incubated with esculin and glucosidase inhibitors is given in red.

This verifies that the presence of scopoletin in the barley leaves is due to the uptake of esculetin (derived from the hydrolysis of esculin) and its subsequent methylation. Moreover, the absence of any coumarinic compounds (Figure 4-45) in the seedlings grown in the medium, in which the hydrolysis of esculin was blocked, unequivocally shows that no transporter capable to catalyze the import of esculin is involved in the uptake of coumarins in barley seedlings.

In analogy to the approaches outlined for barley, also the *Raphnus* seedlings were incubated with esculin in the absence and presence of the glucosidase inhibitors.

Despite the complexity of the chromatograms, it clearly can be stated that the huge accumulation of esculin in the *Raphanus* leaves did not occur when the glucosidase inhibitors had been added to the medium (Figure 4-46). This confirms the finding in the barley seedlings that esculin - as a glucoside - is not taken up and accordingly, no transporter is responsible for the import of esculin described above.

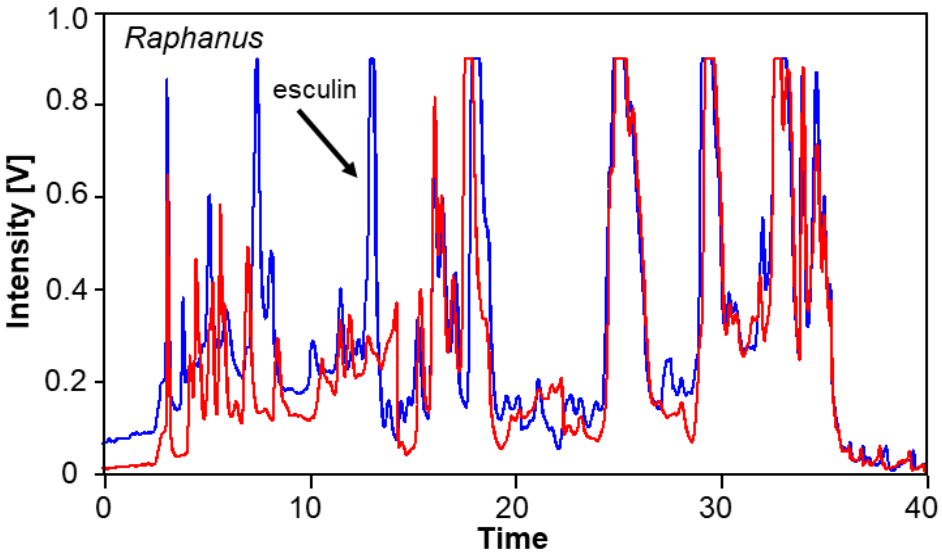

Figure 4-46: Hydrolysis of esculin by glucosidases in radish seedlings (*Raphanus sativus*). Esculin was applied after the application of glucosidase inhibitors into the media. The corresponding HPLC chromatogram of the extract from plants incubated with only esculin is displayed in blue, this of plants incubated with esculin and glucosidase inhibitors is given in red.

Chapter 5: Discussion

In order to get further information on the recently discovered phenomenon of "*Horizontal Natural Product Transfer*", the uptake and modification of natural phenolic compounds were exemplarily investigated employing various coumarins, such as umbelliferone. In this context, various aspects had to be examined. At first, an appropriate hydroponic system for the application of umbelliferone was successfully established, which was used to verify the uptake of umbelliferone by the various tested plant species. Whereas in seedlings of radish, pea and flax the imported coumarins were just accumulated, they were modified in barley and garden cress seedlings. The related derivatives had been identified and the site of modification was elucidated. Moreover, it was shown that cytochrome P450 enzymes are involved in these modifications, and the uptake process of the studied coumarins is passively occurred without the involvement of transporters. In the following sections, these insights are discussed in detail before the relevance of the main results is evaluated with respect to the so-called green liver concept.

5.1 Leaching of endogenous coumarins – an unpredicted cognition when establishing the hydroponic system

The optimized hydroponic system allows the cultivation of seedlings of the different plant species employed in this study. An aeration system using small pipes was introduced, which bubbles filtered air in the medium. As a result, all cultivated seedlings of one batch grow under the same conditions, and thus, the variations caused by the inconsistent conditions were minimized. Every single seedling was held in its place by the means of a circular foam sheet. In consequence, the fed compounds should come in contact only with the roots of the seedlings, whereas any direct contact with the aerial parts was excluded. This assumption should be verified by wrapping small tissue papers around the

seedlings and analyze them - after several days of cultivation – for the occurrence of coumarin. Surprisingly, also in the papers wrapped around the control barley seedlings, small amounts of umbelliferone were detected. This unpredicted cognition - in combination with the finding that the amount of umbelliferone was only slightly enhanced in the tissue papers wrapped around the seedlings grown in the umbelliferone-containing medium - enables two important statements. First, the diffusion of umbelliferone from the coumarin-containing medium via the foam sheets into the shoots could be excluded - an outcome that verifies the suitability of the system for the uptake experiments. Second, the presence of umbelliferone in the tissues wrapped around the control seedlings undoubtedly shows that the endogenous umbelliferone diffuses into the apoplastic space. This vividly validates that coumarins are indeed able to diffuse across biomembranes, a capability which could be self-evidently predicted by their physicochemical properties, i.e., their log*P* values. In this context, it has to be considered that the increased umbelliferone concentration due to its uptake by the roots from the medium and its translocation into the leaves, enhances the coumarin concentration in the shoots. In consequence, the amount of umbelliferone diffusing into tissue paper is enhanced in comparison to the control seedlings.

Indeed, this awareness does not reflect a completely new and seminal realization, but it indicates that we frequently do not really consider that substances, which are accumulated within the plant cells, simply could exit, provided their physicochemical properties enable them to diffuse across membranes.

5.2 Uptake and translocation of coumarins

5.2.1 Uptake of umbelliferone

As mentioned before, coumarins were chosen as a model substance because of their distinctive fluorescence and the simplicity to follow their uptake into the plants, and detect their derivatives, which mostly are also fluorescent.

Umbelliferone represents a phenolic compound, which - in comparison to the other coumarins like esculetin - reveals much higher stability and resistance against oxidation. Accordingly, umbelliferone was chosen as a model compound.

As displayed in the "Results" chapter (section 4.2.1), in the seedlings of all five different species employed in the feeding experiments (barley, radish, pea, flax, and garden cress), huge amounts of umbelliferone were taken up by the roots and accumulated in their aerial parts. Since any contamination by direct contact of the leaves with the media containing umbelliferone could be excluded, these findings clearly show that this coumarin is indeed taken up from the medium by the roots and subsequently translocated into the leaves.

In addition, the detection of derivatized compounds in barley and garden cress grown in the umbelliferone-containing medium does not only reaffirm the uptake of umbelliferone but, also verifies that in seedlings of some plant species the imported coumarin is derivatized. While those transformations require an active metabolism, coumarin must be present within the cells. Despite the difference in the modification patterns of the umbelliferone in various acceptor plants, the occurrence of these coumarins clearly certifies and proves that umbelliferone had been taken up by the roots of all tested seedlings.

As already mentioned, the diffusion of a certain substance across biomembranes is based on its water solubility as well as its hydrophobic properties. The k_{OW} value, representing the distribution coefficient between octanol and water, or its decadal logarithm, i.e., the log k_{OW}, also denoted as log$P$ (Cronin and Livingstone, 2004), is established to be a suitable marker for such diffusion (Trapp, 2000; Trapp and Legind, 2011). All substances revealing a log$P$ value between −1 and 3 can passively penetrate biomembranes (Trap, 2000; Trapp and Legind, 2011).

Since the log$P$ of umbelliferone is about 1.5, the observed uptake of this coumarin is fully in accordance with its chemical properties. Indeed, the fact that simple

coumarins passively penetrate biomembranes, should have already been known from the early work of Werner and Matile (1985) and Tabata et al., (1984). These authors showed that hydroxylated coumarins fed to isolated protoplasts from barley leaves or to suspension cultures of various plant species, respectively, are taken up into the cells, where they are subsequently glucosylated. Accordingly, it was most likely and predictable that umbelliferone would be taken up by all acceptor plants. Nonetheless, it has to be emphasized that up to now, such consideration had never been made for intact plants. Hence, the data obtained in this study represent the first experimental proof for such uptake. This, in turn, demonstrates that the phenomenon of horizontal transfer of natural products is not restricted to alkaloids but also involves phenolic compounds.

5.2.2 Uptake of esculetin

Originally, esculetin was not considered as a model compound to elucidate its uptake and derivatization within plants, since this dihydroxy coumarin is relatively unstable and is oxidized rapidly to a quinone. This was verified by the finding that the roots of the various seedlings, which grew in esculetin-containing media, instantly turned black, due to the oxidation and the subsequent tanning reactions. However, in order to elaborate more information on the biotransformation reactions, several series of experiments employing esculetin had been performed, since esculetin was postulated as one of the main intermediates in the conversion of the imported umbelliferone to scopoletin and esculin in barley and garden cress, respectively.

With respect to diffusion and uptake, esculetin should behave similarly to umbelliferone, since its log*P* value of 1.2 is close to that of the umbelliferone (1.5). However, it turned out that the situation was quite different. In contrast to umbelliferone, no accumulation of esculetin was observed in any of the employed seedlings. In *Pisum* and *Linum*, neither esculetin nor any derivative was found,

while in barley, *Lepidium*, and *Raphanus* indeed various derivatives could be detected, which putatively had been generated from the imported esculetin. Nonetheless, no accumulation of esculetin did occur.

The lack of uptake and accumulation of esculetin could be due to its oxidation, therefore to a decreasing availability in the medium. Moreover, the oxidation of esculetin and the subsequent blackening reactions of the roots could decrease the efficiency of any diffusion. Polymerization of the esculetin-derived quinones forms a pigmented layer similar to melanin, which is well-known from the polymerization of quinones derived from mono- and di-hydroxy phenols (Walker, 1995; Walker and Ferrar, 1995). A corresponding melanin layer is formed in plants in the course of wounding and acts as a physical barrier to prevent further infection by sealing the wounded site. Moreover, melanin is known to absorb many chemicals (Larsson, 1993) and to act as a diffusion barrier (Belozerskaya et al., 2017). In consequence, the generation of the melanin-like black layer around the roots might prevent the diffusion of esculetin. This assumption was verified by employing *Linum* seedlings, which typically accumulate enormous amounts of umbelliferone when growing in umbelliferone-containing medium ("Results" chapter, Figure 4-5). However, when the seedlings had been treated with esculetin two days before applying the umbelliferone, only small amounts of umbelliferone are taken up (Appendix, Figure A-6).

Despite these coherences, it has to be noted that esculetin is taken up by the roots of barley, *Lepidium*, and *Raphanus* seedlings. Consequently, the question arises, why esculetin is taken up by seedlings of certain species while not in others. At first sight, it seems to be obvious that differences in the redox potential, and thus, in the extent and velocity of esculetin oxidation might be responsible for the observed differences. However, this assumption has to be rejected: in order to prevent the browning reaction putatively responsible for the decreased import by

hampering the diffusion of coumarins, ascorbic acid was added as an antioxidant to the medium. Unfortunately, even in the presence of ascorbic acid, the browning reaction in the course of esculetin feeding could not be suppressed, and the uptake of esculetin was not enhanced (Appendix, Figure A-7). Indeed, it could not be fully excluded that the applied ascorbic acid is oxidized in a very short time, and thus, its reducing effect is lost quickly. Nonetheless, it seems to be very unlikely that the putative differences in the redox potential in the rhizosphere of the various seedlings might be responsible for the variations in esculetin uptake.

Another possibility could be due to differences in the impact of esculetin on the metabolism of the seedlings. According to the literature, esculetin inhibits the growth and elongation of the roots of pumpkin seedlings (Hossain et al., 2008), maybe by slowing down the mitosis in the root tissue by decreasing the rate of oxygen uptake by meristematic cells (Kupidlowska, 2001). However, as such growth effects would be relevant only after several days of cultivation. Accordingly, a corresponding impact on the esculetin uptake could be ruled out.

The question remains, why is esculetin is taken up (and derivatized) by seedlings of barley, *Lepidium,* and *Raphanus*, whereas it is not by those of *Pisum* and *Linum*. Indeed, any variation in esculetin uptake could only be due to either difference in the concentration gradient between medium and cells, or to differences in the diffusion resistance, e.g., of the barrier built by the melanin-like browning products as outlined above.

In this context, it has to be emphasized that in all cases the genuine coumarin esculetin is not accumulated, but its derivatives. Thus, seedlings of barley, *Lepidium,* and *Raphanus* take up the esculetin and instantly transform it. Despite the low concentrations of the "un-oxidized" esculetin in the medium, in *Raphanus,* a quite high amount of derivatized compounds is accumulated, whereas in seedlings of barley and garden cress only minor amounts could be detected.

These variances in uptake and derivatization might be explained by the more efficient biotransformation in *Raphanus*, e.g., because the glucosyltransferase responsible for the generation of esculin might be more active and able to glucosylate the imported esculetin efficiently. The rapid glucosylation process and thus, the depletion of the esculetin in the cytoplasm, creates the driving force to take up more esculetin. Such passively driven import of esculetin was already outlined by Werner and Matile (1985) who investigated uptake and glucosylation of esculetin by isolated barley protoplast. The authors reported that the kinetics of its uptake indeed was proportional to the esculetin concentration in the medium.

In comparison to the efficient biotransformation of esculetin in *Raphanus*, the rate of scopoletin and esculin production in barley and garden cress, respectively, is far lower. In consequence, the resulting driving force to import esculetin should be lower, too. Yet, such an assumption would mean that the extent of an esculetin import depends on the efficiency of its derivatization. This seems to be confirmed by the fact that in seedlings of P*isum* and L*inum*, no uptake could be detected. Obviously, the oxidation process is faster and more efficient than the ability of the seedlings to take up the "un-oxidized" esculetin and modify it. Accordingly, the lack of uptake seems to be due to the absence of an appropriate concentration gradient, since the deficiency of modification in the cells does not create a lower concentration of esculetin than that in the medium. However, such a scenario requires that the related derivatization process does occur in the root cells, directly after the esculetin import. In this context, we have to consider that in the case of *Raphanus* and *Lepidium* the modified product esculin is a glucoside, which – in contrast to the corresponding aglycones – cannot diffuse through membranes and will not be translocated via the xylem[3]. Moreover, an alternative translocation of

[3] Indeed, in principle, a translocation of esculin might be realized via xylem as it is known for carbohydrates in budding maple. However, such particular case seems very unlikely. Nonetheless, as a putative translocation via xylem cannot be fully ruled out, and, accordingly, this issue have to be investigated in forthcoming studies.

the putatively generated esculin via phloem seems also to be quite unlikely[4]. In consequence, it cannot be excluded that indeed esculetin, which is taken up by the roots of *Raphanus* and *Lepidium* seedlings – at least to a certain extent - is instantly glucosylated to esculin within the roots. In this case, however, the glucoside would be restrained within the root cells and the outlined "*lack of uptake*" would have to be re-denoted as "*lack of translocation into the shoots*". Nonetheless, according to this assumption, the observed accumulation of this glucoside in the shoots cannot be explained,

In principle, these considerations would also apply for the putative generation of scopoletin in the roots. However, the situation is different since, in contrast to esculin, scopoletin can easily diffuse through biomembranes and would not be restrained in the roots but will be translocated into the shoots via the xylem, driven by the transpiration. Nevertheless, also in this case, the modification of esculetin (to yield scopoletin) has to be very efficient in order to generate an appropriate concentration gradient required for its uptake out of a medium exhibiting a very low concentration of "un-oxidized" esculetin. Indeed, the generation of scopoletin in the roots might be deduced from the fact that as mentioned before ("Results" chapter, section 4.2.1.1) in the case of umbelliferone feeding – at least in part – scopoletin seems to be produced already in the roots. For further elucidation of this complex issue, detailed analyses with respect to the site of modification had been performed ("Results" chapter, section 4.2.1.2) and will be discussed below (section 5.3.2).

[4]. In principle, a transport of esculin via phloem conceivably might occur. However, in this case, an appropriate transporter, which catalyses the loading the coumarin glucoside into the companion cells of the sieve tubes, must be present. In consequence, as outlined for the translocation via xylem, an allocation via phloem cannot be fully ruled out, and further investigation is required.

Overall, there seem to be various possibilities explaining the differences in the extent of esculetin uptake, i.e., variation in the concentration gradient due to differences in the efficiency of derivatization, or the impairment of diffusion due to the melanin-like barrier formed. Indeed, according to the detailed discussion above, it is quite unlikely that the biotransformation processes are the fundamental and leading force for the uptake and import of esculetin. This is underlined by the fact that umbelliferone is taken up by all seedlings, although it is not derivatized in various plant species.

In conclusion and based on the various possibilities and their detailed discussion, it is more likely that the main reason for the lack of esculetin uptake is related to the diffusion barrier of the melanin layer. Depending on the quantity and quality of the corresponding browning and tanning reactions, the diffusion velocity will be affected differently: an extensive manifestation of these processes will prevent any diffusion, while a lesser extent still will allow an uptake into the root cells. Further studies are required to examine this point in deep, i.e., by experiments delivering the esculetin directly to isolated root protoplasts, in which – analogously to the experiments employing mesophyll protoplasts (Werner and Matile 1985) -the intensive browning reactions and thus the generation of a melanin-like barrier will not occur.

5.2.3 Pretended uptake of esculin

The fast oxidation of esculetin and the corresponding lack of its uptake encouraged the employment of a continuous and relatively more stable supply of this coumarin. However, instead of permanently adding new esculetin, an alternative approach was realized, i.e., the application of esculin. This glucoside, when hydrolyzed by β-glucosidases, releases its aglycone esculetin and thereby should provide a continuous supply of esculetin, which could be taken up by the roots. However, this approach requires appropriate hydrolysis of esculin to esculetin,

conjecturally catalyzed by a corresponding β-glucosidase. As β-glucosidases frequently are located in cell walls (Konno et al., 1996), the liberation of esculetin in the direct vicinity of root cells should take place, and thus, an uptake of the coumarin might occur before it is oxidized. The presence of a corresponding β-glucosidase should be confirmed by a simple experiment: when the non-fluorescent 4-methylumbelliferyl-glucoside was added to the culture medium, it indeed was hydrolyzed, visible by the strong fluorescence of the produced aglycone, i.e., 4-methyl umbelliferone. However, the liberation of the aglycone - demonstrated by its bright-blue fluorescence – was distributed uniformly throughout the medium, not only near the plant roots. In consequence, there was no enhanced concentration of (un-oxidized) esculetin in the vicinity of the roots. Accordingly, the same results were attained when esculin was fed to the seedlings as in the case of esculetin application.

As in *Lepidium* and *Raphanus,* high amounts of esculin are accumulated, when esculin is present in the medium, theoretically a direct uptake of esculin cannot be excluded instead of an uptake of the liberated esculetin, which subsequently is re-glucosylated in the acceptor plant. However, due to the strong hydrophilicity of the glucoside (log*P* ~ -1.1), a passive diffusion has to be excluded. Accordingly, a direct import of esculin could only be performed via a transporter. In order to differentiate between both possibilities, i.e., an uptake as esculetin (aglycon) after the hydrolysis of esculin and subsequent re-glucosylation to esculin in the seedlings, or alternatively, an uptake as glucoside (esculin), catalyzed by a transporter, the cleavage of the glucoside in the medium has to be suppressed. For this, the β-glucosidase inhibitors *N*-glucosyl- and *N*-galactosyl-piperidine had been employed, whose effectivity had been proven by De Melo et al., (2006). When esculin was applied together with a mixture of *N*-glucosyl- and *N*-galactosyl-piperidine to the culture medium of barley and *Raphanus* seedlings, neither esculin nor any derivatives accumulated in the seedlings. This finding firmly proves the

inability of an uptake of un-hydrolyzed esculin and verifies two conjunctures: first, the glucoside (revealing a log*P* of -1.1) indeed is unable to pass passively through the membranes, and second, that no transporter in the roots is able to catalyze the uptake of esculin, and consequently is involved in the observed accumulation of esculin in the seedlings. These coherences are confirmed by the findings of Tabata et al., (1984), who applied esculetin to different cell suspension cultures and found the "biotransformed" esculin only in the cultured cells and not in the surrounding culture medium, displaying that the produced glucoside (esculin) cannot diffuse through the plasmalemma out of the cells into the medium.

5.2.4 Translocation of coumarins

The translocation of many xenobiotics (Trapp and Legind, 2011) and other natural products like alkaloids (Selmar et al., 2015a, b; Weidner et al., 2005; Nowak 2017) within the acceptor plants is confirmed to occur via the xylem. The driving force for this translocation is either the transpiration or the root pressure. In consequence, the extent of translocation and thus, accumulation of the substances in the leaves depends on the intensity of both these factors. The same coherences are also relevant for the translocation of the umbelliferone in the employed seedlings, confirmed by its occurrence in the guttation droplets of the barley and garden cress seedlings.

It is worth noting that slight amounts of umbelliferone were also detected in the guttation droplets collected from the control barley seedlings. Obviously, a small amount of endogenous umbelliferone, due to its ability to pass passively biomembranes (Log*P* 1.5), is not withheld in the cells and occurs in the apoplastic space and thus also in the xylem. This situation is vividly confirmed by the finding that endogenous umbelliferone was also detected in the tissue paper wrapped around the control barley seedlings ("Results" chapter, section 4.1). Nonetheless, the concentration of the umbelliferone in the guttation droplets of barley seedlings

fed with exogenous umbelliferone is massively higher in comparison to the controls. In addition, also the modified product in the barley seedlings i.e., scopoletin, is present in the droplets; its concentration is similar to that of umbelliferone. Indeed, in the same manner, as endogenous umbelliferone occurs in the guttation droplets, it has to be assumed that also small amounts of the endogenous scopoletin, as well as that of the scopoletin generated from umbelliferone would be present in the guttation droplets ("Results" chapter, Figure 4-14). However, when considering the ratio of these two coumarins, it has to be stated that the amounts of scopoletin and umbelliferone in the guttation droplets of barley are quite the same, whereas far higher contents of umbelliferone are accumulated in the leaves in comparison to scopoletin. In case of simple diffusion of the accumulated coumarins out of the mesophyll, the concentration of umbelliferone should be far higher than that of scopoletin. Accordingly, it could be assumed that the methoxylation of the imported umbelliferone – at least in part – takes place within the roots. In consequence, also scopoletin is translocated from the roots to the shoots via the xylem, resulting in a much higher ratio of scopoletin to umbelliferone in the guttation droplets in comparison to that of the coumarins accumulated in the leaves. In order to further elucidate this inconsistency, corresponding studies on the site of modification have been performed (see section 5.4.2).

Also in *Lepidium,* the endogenous scopoletin as well as the imported umbelliferone are present in the guttation droplets, and thus in the xylem. However, the concentration ratio of umbelliferone to scopoletin is quite the same as that in the leaves. Accordingly, there seems to be no significant translocation of scopoletin via the xylem, and, in consequence, no generation of scopoletin in the roots of *Lepidium* seedlings. This is confirmed by the fact that in *Lepidium* seedlings, the major product of the derivatization of the imported umbelliferone is esculin. As expected, - because of its negative log*P*-value (-1.1) - this compound is not detected

in the guttation droplets and therefore a putative xylem transport can be ruled out. Furthermore, no esculetin is present in the guttation droplets. Accordingly, the site of the umbelliferone biotransformation, i.e., its hydroxylation to yield esculetin and the subsequent glucosylation to esculin must occur in the leaves, and not in the roots. In contrast, in the case of barley, the situation is ambiguous since modification in roots and allocation of scopoletin into the shoots cannot be excluded comprehensively.

5.3 Modification of coumarins

As outlined in the "Results" chapter, the fate of the imported coumarins was different in the various employed plant species (*Pisum*, *Linum*, *Raphanus*, *Hordeum*, *Lepidium*). Whereas umbelliferone was accumulated in the leaves of all acceptor plants, this coumarin also was modified only in certain plants. Most of the derivatized compounds are synthesized from the fed compounds via hydroxylation, methylation and/or glucosylation reactions. It is worth noting that some of the derivatized compounds could be generated via different intermediates that will finally give rise to the same compound. Apart from the elucidation of the sequence of the various modifying steps also their site of occurrence, i.e., the site of their modification is of special interest.

5.3.1 Hydroxylation, methylation, and glucosylation

In barley, a major share of the imported umbelliferone is converted to scopoletin by methoxylation, and in garden cress, the massive uptake of umbelliferone leads to the generation and accumulation of esculin. Although the end products are different, in both cases, the imported umbelliferone has to be hydroxylated to esculetin, before methylation and glucosylation took place in barley and *Lepidium*, respectively (Figure 5-1).

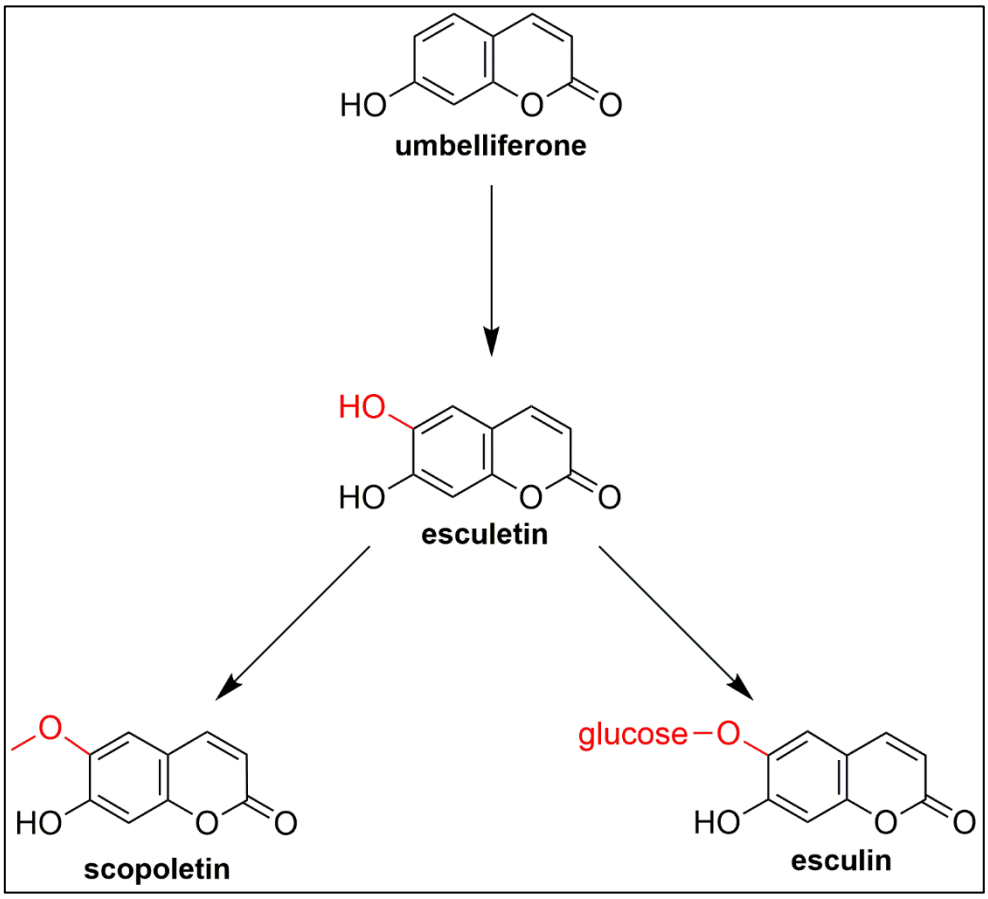

Figure 5-1: In both plant species barley and garden cress, the imported umbelliferone has to be firstly hydroxylated to yield esculetin. Then, methylation and glucosylation are required to produce scopoletin and esculin, respectively.

At the first glance, these basic modifications appear reasonable, and no further investigations seem to be required. However, when looking a little bit closer and realizing that in *Lepidium* additional derivatives are generated from umbelliferone, the situation becomes much more ambiguous. Apart from the large amounts of esculin in *Lepidium* seedlings when fed with umbelliferone, also scopolin is generated ("Results" chapter, Figure 4-10). Moreover, a third derivative, presumably isoscopolin, is produced, too. In this context, it has to be mentioned that with respect to the biosynthesis of coumarins, many options for the order of events (hydroxylation, methylation, glucosylation) are frequently described (Bourgaud et al., 2006). In addition, the question arises, why in the *Lepidium* seedlings, which genuinely contain tremendous high concentrations of umbelliferone, this endogenous coumarin is not modified in the same manner as

the imported ones. Indeed, one explanation might be due to spatial effects, i.e., to differences in the localization of enzymes and substrates. Accordingly, the issue of compartmentation will be addressed in the next section (5.3.2).

The occurrence of scopolin (and maybe also isoscopolin) raises the question, whether or not the sequence of modification indeed is as simple as initially assumed. When comparing the structures of the imported compound umbelliferone and its various derivatives, e.g., esculin and scopolin, there is no doubt that a second OH group has to be introduced. As mentioned above, in the case of esculin, the order of events is quite clear: umbelliferone has to be hydroxylated before the glucose moiety is attached to yield esculin (Figure 5-1). However, it is worth mentioning that the esculetin as an intermediate in the case of *Lepidium* could be glucosylated in two positions, to yield either the 6-OH isomer esculin or the 7-OH isomer cichoriin (Figure 5-2, pathway 3). Nonetheless, as only esculin was detected, the direct esculin production as already outlined in Figure 5-1 seems to be very likely.

In contrast, in the case of scopolin or isoscopolin generation, the situation is much more ambiguous. Indeed, esculetin might be also generated as the first intermediate, which – after methylation to scopoletin (Figure 5-1) is subsequently glucosylated to yield scopolin (Figure 5-2, pathway 2). However, another option also might occur: the produced intermediate, i.e., esculetin, could also be methylated at positions C-7, generating isoscopoletin instead of scopoletin, which subsequently could be glucosylated to isoscopolin (Figure 5-2, pathway 2). In this context it has to be noted that Kim et al., (2008) reported that indeed both the hydroxyl groups of esculetin (at the C-6 and C-7 positions) are targets for *O*-glycosylation, yielding in esculin and cichoriin as well as for *O*-methylation, yielding in scopoletin and isoscopoletin, respectively. The later ones subsequently could be glucosylated to scopolin and isoscopolin, respectively.

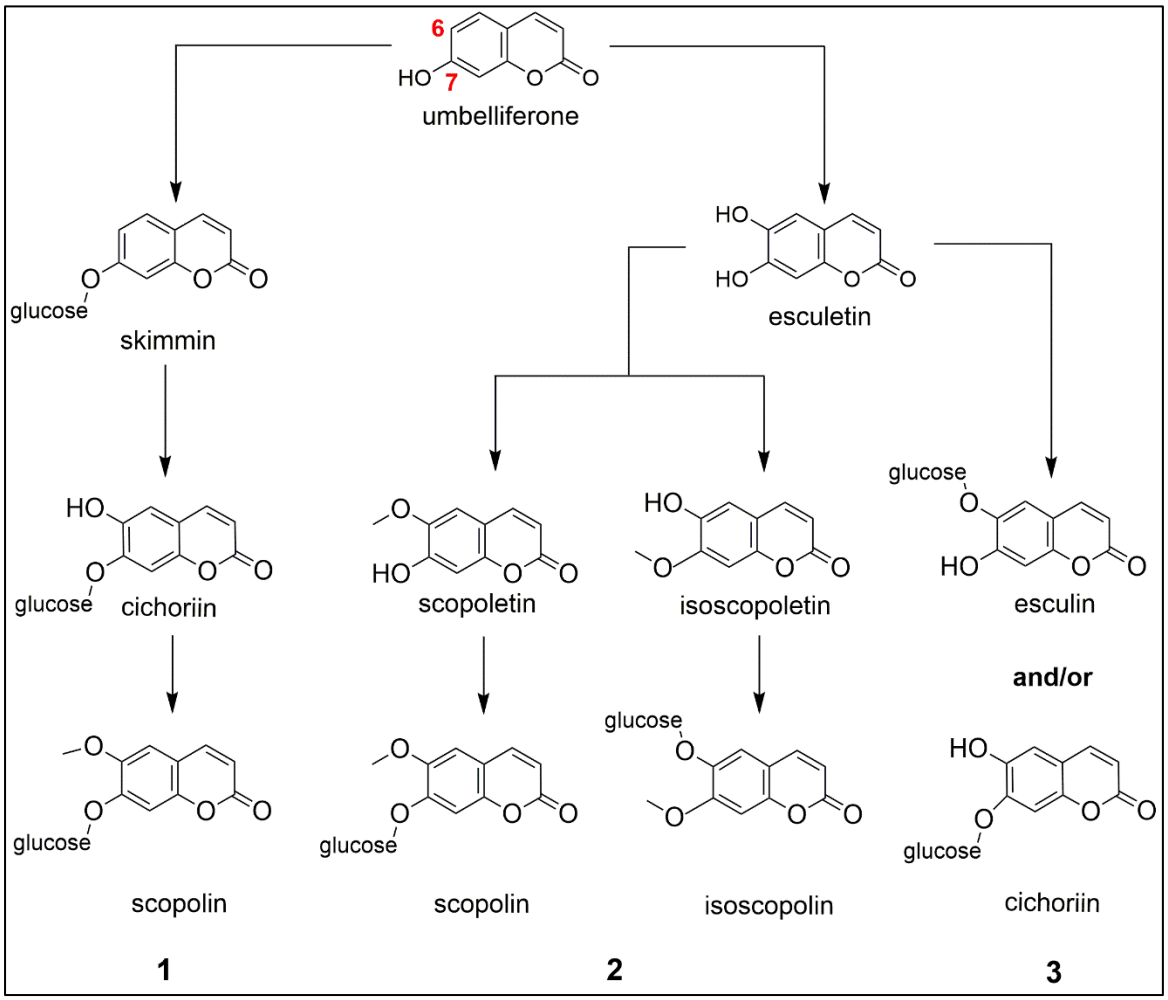

Figure 5-2: Alternative options for the generation of the various derivatives of imported umbelliferone.

Alternatively, scopolin might also be produced from umbelliferone by changing the order of events, i.e., by glucosylation prior to hydroxylation, resulting in skimmin, which subsequently has to be hydroxylated and methylated to yield scopolin (Figure 5-2, pathway 1). Actually, the related compound, denoted as skimmin, is described to occur in *Saussurea hieracioides* together with scopolin and umbelliferone (Tan et al., 2014). However, in *Lepidium* acceptor plants, no skimmin was detectable. This indeed could be due to the fact that the hydroxylation of skimmin (and the subsequent methylation) might efficiently be catalyzed by enzymes exhibiting a very high affinity to skimmin. As consequence, only very small concentrations of the putative intermediate skimmin would be present.

It is worth considering that the putative isoscopolin can only be produced from esculetin, but not from skimmin since the 7-OH group is blocked by the attached glucose moiety. Due to these considerations, also the formation of scopolin via esculetin as an intermediate seems to be more probable than via skimmin. This assumption is supported by the finding that the concentration of the postulated intermediate esculetin is indeed enhanced in the seedlings of *Lepidium* grown in umbelliferone-containing media.

In order to further elucidate this issue, in particular the putative intermediate, i.e., esculetin, was applied to seedlings. In case of scopolin generation via skimmin, the production of scopolin – in relation to the generation of esculin – should be massively reduced when applying esculetin instead of umbelliferone. However, in *Lepidium* no substantial difference in the ratios of esculin to scopolin in acceptor plants was detected, when fed either with umbelliferone ("Results" chapter, Figure 4-10) or with esculetin ("Results" chapter, Figure 4-36), respectively. Thus, skimmin as an intermediate in scopolin production can be ruled out.

In barley, the major share of the imported esculetin is methylated to scopoletin. This strongly supports the assumption of bio-transformations order described in Figure 5-1. In addition, in barley seedlings, also small amounts of esculin and scopolin were detected. Indeed, these derivatives have to be generated by the glucosylation of their corresponding aglycones, i.e., of esculetin after its import into the seedlings, or of its methylated derivative, i.e., scopoletin, respectively. In the same manner, esculin in *Raphanus* is generated by the glucosylation of esculetin.

In this context, it has to be mentioned that the putative intermediate, i.e., esculetin, could not be detected in the seedlings although its putative derivatives are accumulated to a quite high extent (see above). Obviously, the enzymes responsible either for the methylation or the glucosylation of esculetin exhibit a

high affinity for this substrate, and accordingly, the steady-state concentration of this substrate is quite low.

The data related to the observed modifications of coumarins are in accordance with the literature. It is well established that coumarins, which are added to the medium of plant suspension cultures are modified within cells. In this context, Tabata et al., (1984) showed that esculetin is taken up by cell suspension cultures of *Lithospermum erythrorhizon, Gardenia Jasminoides,* and *Nicotiana tabacum* and subsequently is glucosylated to esculin. Moreover, Werner & Matile (1985) reported that protoplasts of barley mesophyll cells take up esculetin and scopoletin from the medium and bio-transform them to the corresponding glucosides, which, subsequently, are accumulated in the vacuoles. Interestingly, apart from esculin (esculetin-6-*O*-glucoside) also nearly equal amounts of cichoriin (esculetin-7-*O*-glucoside) are formed. However, it has to be emphasized that Werner and Matile (1985) detected as derivatives of the imported coumarins predominantly their glucosides, i.e., esculin and cichoriin, respectively. But, it has to be noted that these glucosides are formed in less than one hour after the esculetin is applied exogenously to barley protoplasts. In contrast, when esculetin is applied to barley seedlings – as shown in the "Results" chapter, mainly the methylated esculetin, i.e., scopoletin, was generated, whereas only very tiny amounts of esculin were produced. Moreover, no cichoriin could be detected at all. Accordingly, the question arises: why the major share of esculetin is (taken up by the roots and translocated into the leaves) methylated to yield scopoletin rather than being glucosylated to generate the glucosides as reported by Werner and Matile (1985). Actually, it could be assumed that the methyltransferase has a far higher affinity for esculetin than the related glucosyltransferase. But, this would also apply in the case of the isolated protoplasts. Accordingly, there must be another explanation. There is no doubt that the generation of glucosides is due to active glucosyltransferases. Accordingly, in the isolated protoplasts, related enzymes

have to be active, whereas in the barley seedling they are not. Indeed, it is well known that glucosyltransferases can be induced by stress, especially in response to pathogens attack (Chong et al., 1999; Langlois-Meurinne et al., 2005). However, in comparison to the generation of phytoalexins in response to the stress, the related induction of glucosyltransferase is delayed. In consequence, initially, the phytoalexins, e.g., scopoletin are formed as protective agents, which- in case of a successful defense against the pathogens - will be glucosylated by the glucosyltransferase, which in turn was induced with a time lag. Subsequently, the resulting glucosides are stored in the host cells and may act as phytoanticipins for forthcoming infections (Chong et al., 1999).

Thus, with respect to the protoplast prepared by Werner and Matile (1985), we have to consider that these cells are also stressed: in the course of their preparation, the cell wall is degraded, and accordingly, many fragments are generated, which might act as elicitors - analogously to the situation of a pathogen attack. In conclusion, in the barley protoplasts, elicitation of glucosyltransferases might have already occurred and the imported coumarins are instantly glucosylated, whereas, in the seedlings fed with esculetin, the glucosyltransferases are not induced and – and in consequence - the coumarins remain as aglycones.

Alternatively, apart from the activation of glucosyltransferases, there might be a further option to explain the observed differences in the modification of imported coumarins, i.e., a spatial compartmentation of substrates and enzymes, i.e., the localization of the hydroxylated coumarins and the glucosyltransferases capable to attach the glucose moiety. However, in this context, we have to consider that the coumarins easily can pass biomembranes, and accordingly, the classical compartmentation rule based on the inability of substances to diffuse across membranes will not account.

In order to further elucidate this complex issue, additional analyses with respect to the site of modification had been required.

5.3.2 Site of modification

The simplest technique to investigate the site where the related modifications take place, i.e., in the roots or the shoots, is by analyzing the xylem sap. For this, an appropriate method is the collection of guttation droplets and studying the nature of the translocated compounds within. As outlined, the analysis of guttation droplets of *Lepidium* revealed that only the genuine umbelliferone taken up by the roots putatively occurs in the xylem, but not its modified products, i.e., esculetin, esculin or scopolin, respectively. Thus, these compounds are not allocated from the roots into the shoots. Furthermore, as discussed above, a translocation via phloem seems to be very unlikely (see page 76). In consequence, it could be assumed that the esculin accumulated in the *Lepidium* leaves is generated after the translocation of umbelliferone into the leaves.

In barley seedlings, the substance accumulated in the leaves as a result of umbelliferone application to the roots is scopoletin. In contrast to the glucoside esculin, scopoletin could be translocated from roots to shoots, in the same manner as umbelliferone. Accordingly, the modification of umbelliferone might already occur in the roots before the resulting scopoletin is translocated into the leaves. As mentioned above, this assumption seemed to be verified by the finding that – in addition to umbelliferone – also scopoletin was present in the guttation droplets, and thus, putatively in the xylem sap. But, the situation became ambiguous, when realizing that - at least in *Lepidium* – endogenous scopoletin is present in the guttation droplets of control plants, too. As the biosynthesis of (endogenous) coumarins is known to take place in the leaves (Zhao et al., 2015), and thus no coumarin transport from the roots to the shoots should occur, it seems to be unlikely that the scopoletin present in the guttation droplets of the *Lepidium* control

plants is due to its occurrence in the xylem. Together with the finding that umbelliferone is present in filter papers wrapped around the shoots of control barley plants (see above), it is obvious that the coumarins do easily diffuse within the leaves across the biomembranes. Accordingly, the scopoletin present in the guttation droplets of the barley and *Lepidium* acceptor plants, which had been fed with umbelliferone, could either be due to scopoletin translocated via xylem or, to the diffusion of scopoletin, which resulted from the modification of umbelliferone in the leaves. Thus, the presence of scopoletin in guttation droplets is not inevitably verifying its occurrence and translocation in the xylem. In consequence, no solid statement could be given, whether or not scopoletin is translocated in the acceptor plants from the root to the shoots, and thus, on the site of umbelliferone modification.

However, despite these coherences, there is a reliable indication that at least a share of the scopoletin accumulated in the barley leaves, is generated already in the roots. As discussed above, in case of a simple diffusion of scopoletin and umbelliferone from the leaf cells into the guttation droplets, the ratio of their concentrations in the droplets should be the same as that one in the entire leaves. Accordingly, the concentration of umbelliferone should be far higher than that of scopoletin. But, in the guttation droplets of barley, this ratio was quite the same ("Results" chapter, Figure 4-14). Thus, after all, scopoletin seems to be present in the xylem. Consequently, it could be assumed that the methoxylation of the umbelliferone, which was imported by the roots of the barley acceptor plants – at least in part – takes place already within the roots.

In contrast, as mentioned above, it seems to be very likely that the situation is different in *Lepidium*, where the modification of umbelliferone, which results in the generation of esculin takes place exclusively in the leaves. Unfortunately, as outlined, for barley, neither a final clue nor a clear indication allows an

unequivocal statement concerning the site of modification. Accordingly, another approach had been employed to further elucidate this aspect by incubating exclusively either just roots or leaves of barley and *Lepidium* seedlings in umbelliferone-containing media.

The corresponding experiment with cut leaves and roots of *Lepidium* ("Results" chapter, Figures 4-16; 4-17, respectively) nicely shows that in both organs umbelliferone is mainly derivatized to yield esculin. Moreover, the pattern of the imported and derivatized coumarins (umbelliferone and esculin, respectively) in isolated roots and leaves is quite similar to that present in the entire seedlings, whose roots had been grown in umbelliferone containing media. From this, at the first glance, it might be deduced that the esculin accumulated in the leaves could result from both options, the modification of umbelliferone in the roots and a corresponding transfer of the modified product, or alternatively, the translocation of umbelliferone into the leaves, where it is converted to esculin. However, since the translocation of the glucosides via xylem can be excluded and via phloem seems to be very unlikely (see above), we have to conclude: a share of the imported umbelliferone is hydroxylated and subsequently glucosylated in the roots, where the resulting esculin is restrained. Another share is translocated via xylem into leaves, where it is converted to esculin, which then is accumulated.

In contrast to the *Lepidium*, in excised barley roots, no modification of umbelliferone was detectable. However, in the leaves, the modification occurred to a very high extent. This clearly shows that the umbelliferone taken up by the roots of the barley seedlings is translocated into the leaves, where it is modified.

Surprisingly, the pattern of the modified products in cut leaves of barley incubated in the umbelliferone-containing medium is markedly different from that of plants, which had been grown as entire seedlings in this medium (Figure 5-3). When cut barley leaves are incubated in umbelliferone-containing solutions, high concentrations of glucosides are accumulated; above all massive amounts of esculin and ample

quantities of scopolin (Figure 5-3, B). This completely is in contrast, to the pattern of coumarins found in the leaves of seedlings that had been grown in umbelliferone-containing media, where only scopoletin was accumulated as a product of modification and no glucosides could be detected (Figure 5-3, A).

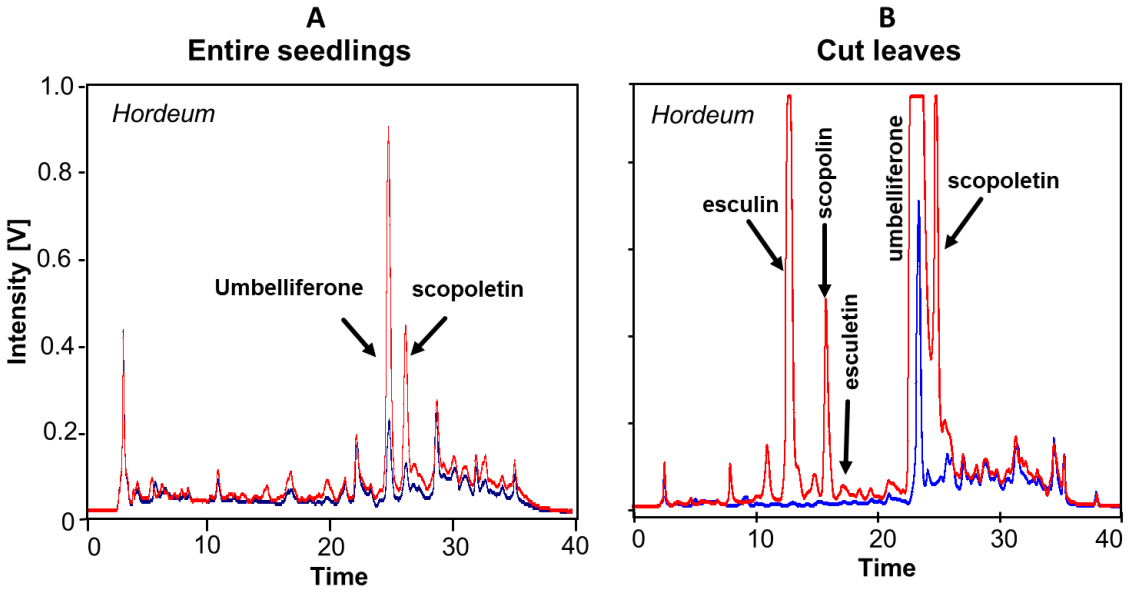

Figure 5-3: The differential patterns of umbelliferone derivatives in the entire seedlings (A) and the excised barley leaves (B). The glucosides, scopolin and esculin occurred only in the cut leaves, where only scopoletin was generated in the entire seedlings.

Indeed, the overall concentrations of imported and derivatized coumarins are far higher in the leaves directly incubated in umbelliferone solution in comparison to that of the entire seedlings. As the amount of the dry material used in both experiments had been nearly the same, a variation of the reference magnitude can be excluded. Thus, putative explanations for this phenomenon must have another basis. In this context, it has to be considered that the concentration of umbelliferone in leaves that had been directly incubated with umbelliferone is far higher than that in the leaves from seedlings whose roots have taken up the umbelliferone. Nonetheless, higher concentrations of umbelliferone would result in the same pattern of derivatives. Since the proportion of glucosidic derivatives, i.e., esculin and scopolin, is massively enhanced in the leaves directly incubated with umbelliferone (Figure 5-3, B), this option has to be ruled out, too. In consequence,

the reason for the variation in the modification pattern might be related to spatial effects.

In this context, we have to consider that in the entire seedlings umbelliferone is translocated via the xylem to the shoots and subsequently diffuses from the interior to the exterior regions. In contrast, when umbelliferone is taken up through the epidermis of the cut leaves, the subsequent diffusion is reverse, from exterior to interior regions. In order to verify the assumption that spatial effects are responsible for the differences in the pattern of umbelliferone derivatives, a further experimental approach was performed: the position of cut leaves in umbelliferone-containing media had been varied. In one case, only the cut ends of the leaves had been in direct contact with the medium, simulating the translocation of umbelliferone through the xylem, as realized in the entire seedlings (Figure 5-4). In another case, only the leaves tips were immersed in the umbelliferone solution (Figure 5-5), which allows only an uptake of umbelliferone by diffusion through the epidermis. In consequence, in the first case, only scopoletin should be generated from the imported umbelliferone (as in the case of entire seedlings Figure 5-3, A), whereas in the case of the immersed tips also the glucosides esculin and scopolin should be produced (as in the case of incubation of cut leaves, Figure 5-3, B). However, contrary to this expectation, the same pattern of derivatives was found in both approaches (see below).

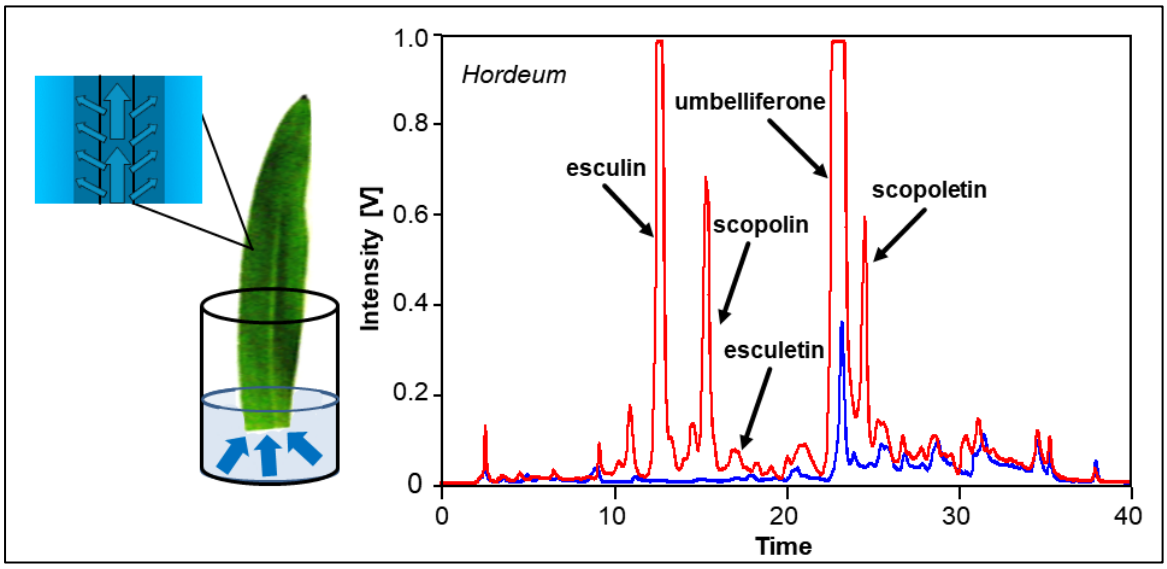

Figure 5-4: Translocation of umbelliferone from the medium into the excised barley leaves only through their cut ends (left), and the pattern of the derivatized compounds in this situation (right).

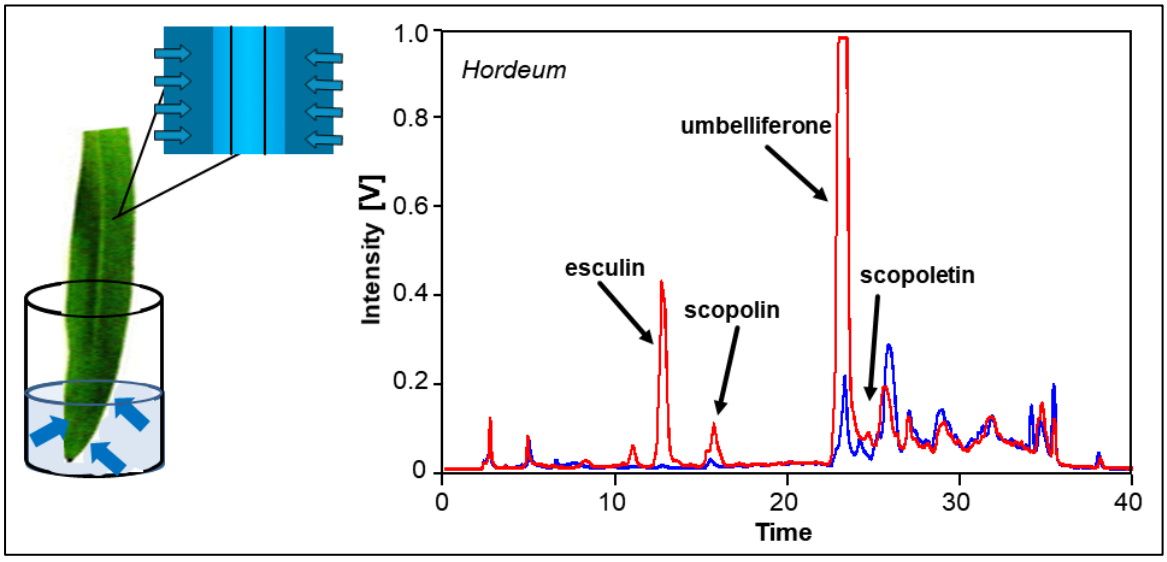

Figure 5-5: Translocation of umbelliferone from the medium into the excised barley leaves through their tips (left), and the pattern of the derivatized compounds in this situation (right).

Thus, the direction of translocation makes no difference, and a classical spatial effect can be ruled out. Anyhow, in one case, the coumarins are glucosylated and in the other are not. As any spatial effect can be excluded, the reason for the observed variation could only be attributed to massive differences in the activity of the glucosyltransferases involved.

The only tremendous difference with respect to the applied umbelliferone is given by the extent of its accumulation within the leaves of both approaches. Consequently, a concentration effect has to be involved, which was not considered so far. Obviously, the high accumulation of umbelliferone and/or its modified products in the leaves induce the activity of the glucosyltransferases that act on scopoletin and esculetin. Indeed, the elicitation of glucosyltransferases by exogenously applied coumarins is well known: when Campos et al., (2019) incubated tomato plants with exogenous scopoletin, esculetin, or umbelliferone, respectively, they found a rapid induction of the glucosyltransferase gene *Twi1* expression within 24 hours. Moreover, other natural products also reveal to induce glucosyltransferase activity: when naphthols are applied to tobacco cells, they also induce glucosyltransferase activity (Taguchi et al., 2003).

Actually, the induction of glucosyltransferase is well established in many host-pathogen interactions. When pathogens attack host cells, due to the elicitors generated by the lysis of cell walls, phytoalexin production is induced (Giesemann et al., 2008; Ahuja et al., 2011). In many cases, the expression of glucosyltransferase genes is also up-regulated (Chong et al., 1999; Langlois-Meurinne et al., 2005). Yet, when looking at the time pattern, it becomes obvious, that – at least in some cases – the induction of glucosyltransferase is somehow delayed. In consequence, when scopoletin is produced as a phytoalexin, the accumulation of its glucoside scopolin is markedly retarded (Shimizu et al., 2005). An obvious explanation for this delayed glucosylation frequently is based on the toxicity of the phytoalexins: when the pathogens are successfully fended, the host cells detoxify the phytoalexin by its glucosylation (Gachon et al., 2004; Gachon et al., 2005).

These coherences suggest that in addition to the "classical elicitation" by fragments of the cell wall, also the presence of high concentrations of putative phytoalexins is responsible for the induction of their glucosylating enzymes. Indeed, the occurrence of both options of elicitation could explain the data elaborated in this

thesis as well as those published in the literature. In this sense, the glucosylation of coumarins in barley protoplasts observed by Werner and Matile, (1985) should be based on the induction of glucosyltransferases by the elicitors liberated in the course of protoplast preparation. On the other side, the increase of glucosyltransferase activity in the barley leaves incubated in umbelliferone solution should be due to an elicitation by coumarins.

These findings open quite new doors in understanding the elicitation of phytoalexins and their subsequent detoxification. Accordingly, there are many novel approaches required to elucidate this metabolic syndrome. Based on the knowledge that – on the one hand - glucosyltransferases are induced in response to pathogen attack (Giesemann et al., 2008; Ahuja et al., 2011), wounding (O'Donnell et al.,1998), or by various components of signaling transfer (e.g., salicylic acid, Horvath and Chua 1996; methyl jasmonate, Imanishi et al., 1998), and – on the other hand - by exogenously added compounds (Campos et al., 2019; naphthols, Taguchi et al., 2003) corresponding investigations are required, in which either classical elicitors, coumarins, or combinations of them should be employed to induce glucosyltransferases. In this context, special emphasis should put on the time-dependent changes in the expression of the glucosyltransferase genes as well as on their activity against various substrates.

Furthermore, the induction of the glucosyltransferases by coumarins nicely explains why – in contrast to the barley seedlings - in the whole *Lepidium* seedlings fed with umbelliferone the glucosides are accumulated, too. Since in the *Lepidium* leaves the concentration of the endogenously accumulated umbelliferone and scopoletin are already quite high, its enhancement by the exogenous umbelliferone (imported by the roots), obviously is sufficient to induce the glucosyltransferases. In consequence, the related glucosides, above all esculin, are generated ("Results" chapter, Figure 4-10). Yet, although the esculetin concentration is far lower than

that of scopoletin (more than tenfold), the amount of produced esculin is massively higher than that of scopolin (more than fivefold). This strongly implies that the affinity of the glucosyltransferase is far higher for esculetin than for scopoletin.

In the same manner, the glucosyltransferase in excised barley leaves seems to reveal a much high affinity toward esculetin than to scopoletin, since also in this plant the ratio of esculin to esculetin seems to be far higher than that of scopolin to scopoletin ("Results" chapter, Figure 4-19). In consequence, the esculetin generated from umbelliferone is converted instantly to esculin, whereas large amounts of scopoletin are not glucosylated. However, when considering the fluorescing properties of the various coumarins, esculetin turns out to exhibit a lower fluorescence than the other studied coumarins. In consequence, when calculating the molar ratio of the coumarins, the ratio of scopolin to scopoletin (0.96) is higher than that of esculin to esculetin (0.27).

In contrast, the affinity of the glucosyltransferase from barley for esculetin seems also to be slightly higher than that of the methyltransferase, since the concentration of esculin is quite higher compared to its methylated derivatives scopoletin and scopolin. In consequence, due to these efficient glucosylation and methylation processes, esculetin is not accumulated in the cut leaves.

5.3.3 Involvement of P450 Enzymes in the biotransformation reactions

The oxidative conversion of many secondary metabolites is frequently catalyzed by cytochrome P450 enzymes (Furge and Guengerich, 2006). Unfortunately, the determination of the activity of these enzymes is quite problematic, since cytochrome P450 enzymes require close collaboration with an appropriate NADP-reductase and the availability of the specific substrates. Therefore, an alternative approach to verify the involvement of P450 enzymes was employed, which is based on the reduction of enzyme activity by application of appropriate inhibitors or competitive substrates,

respectively. A common and accessible inhibiting agent is naproxen (Miners et al., 1996).

Actually, in seedlings of both, barley and garden cress, naproxen strongly reduced the generation of the derivatized coumarins, when applied together with the umbelliferone. Accordingly, it could be deduced that naproxen inhibits the enzyme activity responsible for the derivatization.

In garden cress, the presence of naproxen significantly decreases the production of the two glucosides, i.e., esculin and scopolin. Moreover, the concentration of the intermediate esculetin slightly decreased. If the inhibiting effect of naproxen would be due to inhibition of the glucosyltransferases, the concentration of esculetin should increase. In contrast, the inhibition of the enzyme catalyzing the hydroxylation of umbelliferone (Figure 5-6) should decrease the concentration of esculetin significantly. However, as this concentration already is very low, putatively due to the high affinity of the glucosyltransferases, the steady-state concentration of the intermediate esculetin will decrease only slightly. In consequence, it could be deduced that the strong naproxen-related reduction in umbelliferone modification is due to the inhibition of its hydroxylation. This confirms the assumption that the hydroxylation of the imported umbelliferone is catalyzed by a cytochrome P450 enzyme. This is in accordance with the literature, i.e., many hydroxylases introducing OH-groups into phenylpropanoid derivatives indeed belong to the cytochrome P450 enzyme family (Bolwell et al., 1994).

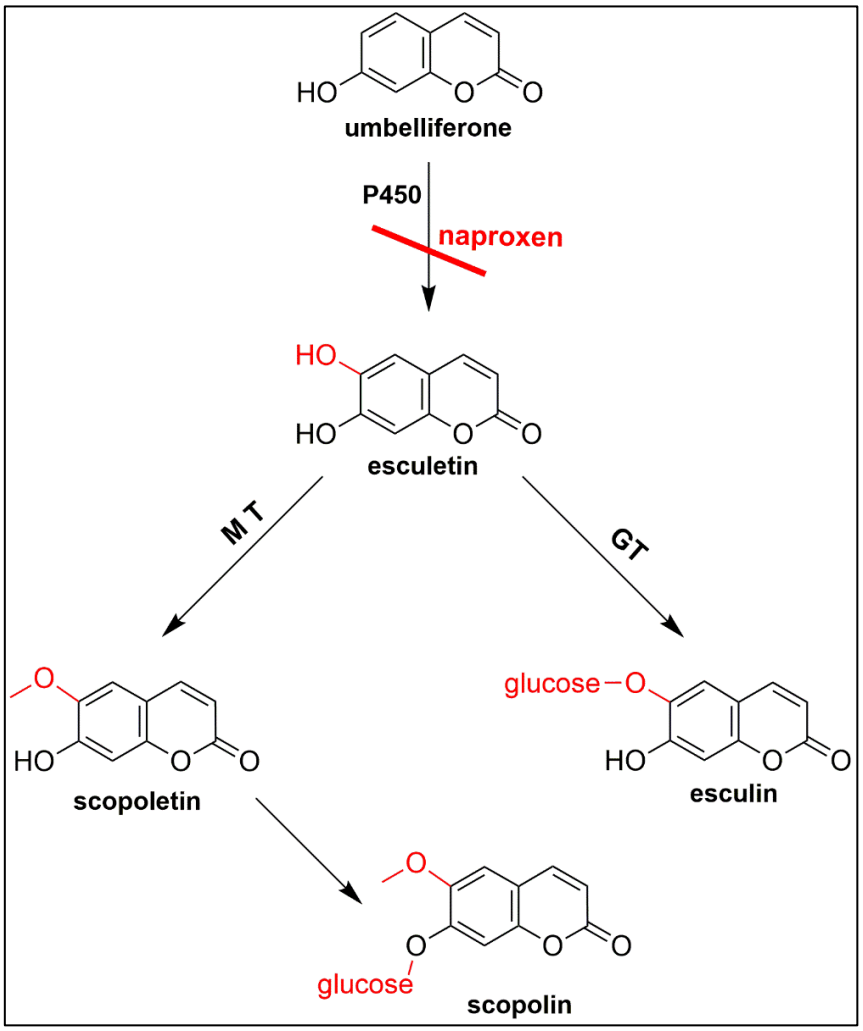

Figure 5-6: Naproxen inhibits P450 enzymes responsible for the hydroxylation of umbelliferone, causing a strong reduction in esculin and scopolin production in garden cress.

In barley seedlings, the final product of modification is scopoletin. Accordingly, two steps are required to convert umbelliferone to scopoletin. In consequence, two enzymes are involved, one catalyzing the hydroxylation of umbelliferone to esculetin and another one for the subsequent methylation of esculetin to yield scopoletin. When barley seedlings are fed simultaneously with umbelliferone and naproxen, the scopoletin concentration is far lower than that in the approaches without this inhibitor. Based on the coherences outlined for *Lepidium* seedlings, the strong reduction might also be explained by the related inhibition of the corresponding hydroxylases, suggesting that also in barley the hydroxylation is performed by a P450 enzyme, which is naproxen sensitive.

However, this deduction has to be abolished, when evaluating the unexpected results obtained from the experiments employing cut leaves. When naproxen was applied simultaneously with umbelliferone to the leaf blades, the production of the esculin was not affected at all, whereas the production of scopoletin and its glucoside scopolin had been reduced significantly. Obviously, the hydroxylation of umbelliferone to esculetin is not inhibited by naproxen, whereas the methylation is (Figure 5-7).

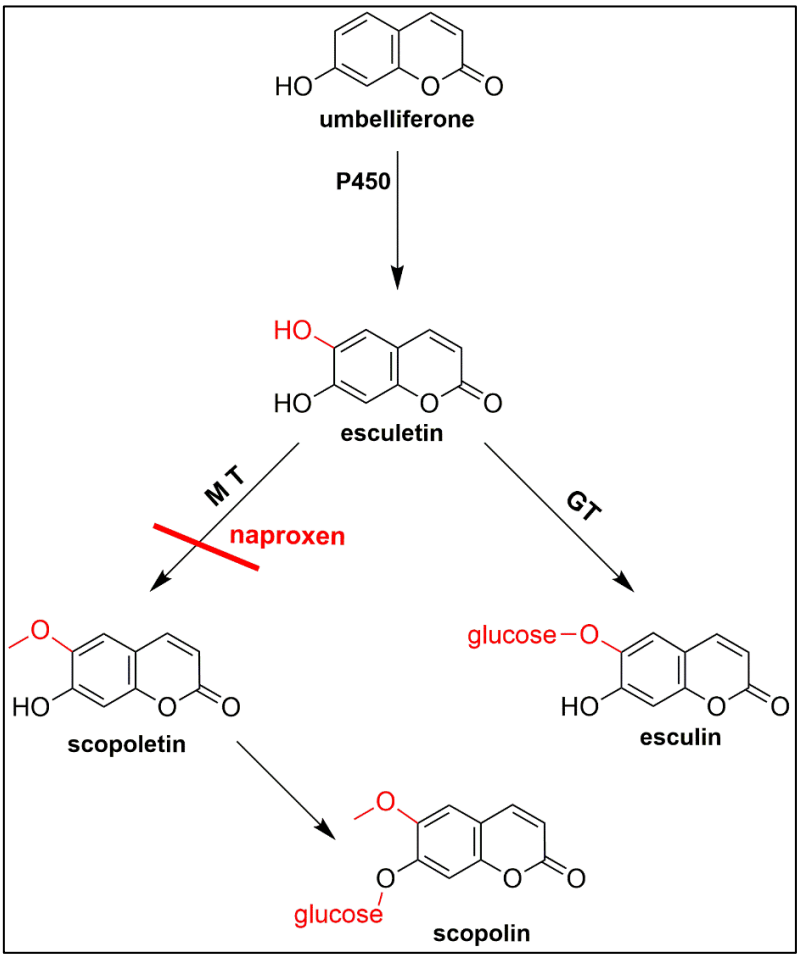

Figure 5-7: Naproxen inhibited methyltransferase enzyme responsible for the methylation of esculetin, causing a strong reduction in scopoletin and scopolin production in barley.

A similar inhibitory effect of naproxen on methyltransferases is already known from the work of Oselin and Anier (2007). Moreover, it has to be stated that the inhibitory effect of a certain substance on various P450 enzymes may strongly differ (Lynch and Price, 2007). Accordingly, the lack of inhibition by naproxen does

not verify that the related enzyme indeed is not belonging to the P450 family. Only the positive finding, i.e., a naproxen-induced inhibition of a certain enzyme, indicates its P450 nature.
In consequence, in contrast to *Lepidium*, no statement could be drawn, whether or not the hydroxylation of umbelliferone in barley is catalyzed by a P450 enzyme.

In conclusion, the employment of a certain inhibitor to identify relevant enzymes of a pathway will not always be as effective as expected, since the inhibitor – as outlined for naproxen – might act differentially in different plant species.

5.4 Differences in the modification patterns among plant species – promiscuous enzymes

As mentioned above, in barley the umbelliferone is converted to scopoletin, whereas in garden cress, it is derivatized to esculin. In contrast, in pea, flax, and radish, no modification could be observed at all. Logically, the question on the cause of the quite different fate of the imported substances in the various plant species arises. Indeed, it is well known that plants, which are exposed to various foreign substances, such as herbicides or veterinary medicines, take up[5] these so-called xenobiotics and modify them (Coleman et al., 1997; Coleman et al., 2008; Bártíková et al., 2015). According to the "Green Liver Concept", these modifications are part of "deliberate" biotransformation processes "in order to detoxify" these compounds. These reactions include hydroxylation, oxidation, or reduction reactions (phase I) and subsequent conjugation processes (phase II). Finally, the derivatives are excreted and deposited in certain compartments (phase III) i.e., vacuoles or apoplastic space (Sandermann, 1994). Yet, in this context, it has

[5] The only prerequisite for this uptake is the ability of the compound to pass the biomembranes, which can be estimated from the log*P* value. All substances revealing a log*P* value between - 1 and 3 are able to passively cross the biomembranes (Trapp, 2000; Trapp and Legind, 2011; Limmer and Burken, 2014).

to be noted that the mode of modification may vary between different plant species (Schulz and Wieland, 1999; Shajib et al., 2012). This is underlined by the selective response of various plants to the same herbicides (Devine et al., 1993; Cole, 1994): whereas resistant plants are able to modify certain herbicides and thereby preventing their effects, susceptible plants just take up and accumulate the toxic substances, which, in turn, massively impact their viability. These differences are attributed to the fact that most of the modifications are catalyzed by cytochrome P450 enzymes, which are known to occur in multiple isoenzymes (Timmerman, 1989; Donaldson and Luster, 1991) exhibiting stringent specificities for different substances. Accordingly, various herbicides are detoxified in a certain plant, whereas other plants are not able to modify these compounds.

Such differences in the ability to detoxify xenobiotics do not really support the assumption that plants have a general detoxification system for a huge variety of xenobiotics as outlined by the "Green Liver Concept" (Sandermann, 1992; Sandermann, 1994). In this context, it has to be considered that herbicide resistance according to the "*Green Liver Concept*" could not have evolved by classical evolution processes. These adaptations would be far too slow to generate appropriate mutations to adapt the detoxification mechanisms in response to certain herbicides, which had been introduced only a century ago. In contrast, the well-known herbicide resistances evolved in only few decades, are due to the fact that only a single mutation in the binding sites for a certain herbicide is required: many herbicides inhibit photosynthesis by competing with plastoquinone at its binding site on the D1 protein. In this case, just a point mutation is sufficient to prevent the binding and thus to generate resistance[6]. In contrast, the selection and

[6] Just a point mutation in the psbA gene encoding the D1 protein causes a Ser-264- Gly amino acid substitution in the PQ binding site, is capable to inhibit the binding of these herbicides to D1 protein to prevent the photosynthesis (Goloubinoff et al., 1984; Hirschberg and McIntosh,1983).

adaptation of a complex detoxification system would require much more time. The skepticism in the existence of a general detoxification system according to the "Green Liver Concept" is underlined by the results presented in this thesis.

The massive differences in the ability of the various plant species employed in this investigation to modify substances imply that the xenobiotics[7] - here represented by umbelliferone - accidentally i.e., just by chance, are modified by enzymes already present in the plants. Accordingly, in the seedlings of some species, appropriate enzymes are present, whereas in others they are not. As all enzymes responsible for primary metabolism are more or less ubiquitous present in all plants, the enzymes responsible for the observed differences in the modification of umbelliferone – or other xenobiotics – must be involved in secondary metabolism.

Secondary metabolism varies from species to species, and even the biosynthesis of the same compound could also differ significantly, i.e., a certain compound could be biosynthesized by various pathways. This in particular accounts for coumarins and their derivatives: in tobacco and in *Hydrangea macrophylla*, ferulic acid is directly converted to scopoletin. Thus, the methylation precedes the cyclization step (Fritig et al., 1970; Kindl, 1971). In contrast in *Daphne mezereum*, esculetin is the putative precursor for the synthesis of scopoletin, and ferulic acid as a precursor of scopoletin can be ruled out (Brown, 1986). Even more controversial are the findings with respect to the order of hydroxylation and glucosylation (Brown, 1962b). In *Nicotiana tabacum,* the biosynthesis of scopoletin involves its glucoside, i.e., scopolin acts as an intermediate, whose glucose already is attached to ferulic acid. Consequently, the methylation step precedes

[7] By definition, xenobiotic are substances that are foreign to life (Patterson et al., 2010). Since natural products are also taken up and modified by the acceptor plants, as for xenobiotics, a new comprehensive broadened definition for xenobiotics is required to include also the substances from other plants, which might be considered as "foreign" to the acceptor plants.

glucosylation. In contrast, when these coumarins are fed to plants they were glucosylated to their corresponding glucosides (Werner and Matile, 1984).

Considering these variations in the biosynthetic pathways and assuming that the related enzymes might be involved in the accidental conversion of imported xenobiotics, such as umbelliferone. Accordingly, it seems to be obvious that the observed modification of xenobiotics and their different markedness in various plants is due to the accidental modifications by enzymes genuinely present in the acceptor plants, where they are involved in secondary metabolism, specific for the particular species. Consequently, the absence of any derivatized product in other plants is due to the fact that appropriate enzymes are missing.

These coherences are in accordance with Parkinson and Ogilvie (2008), who reported that the detoxification reactions are performed by enzymes revealing a quite broad substrate specificity. However, this conjecture seems to contradict our understanding of enzyme-substrate specificity, frequently depicted by the lock and key model, especially with respect to the position and the stereochemistry of the functional groups involved (Heller and Forkmann, 1988; 1993). This paradigm was established and manifested especially with respect to enzymes involved in secondary metabolism by claiming their very high substrate specificity (e.g., Hartmann, 1996; Wink, 1997). Meanwhile, we are aware that the substrate specificity of enzymes is far lower than initially assumed (Atkins, 2015).

In consequence, the feature of "high substrate specificity" is replaced by the indication of promiscuous enzymes. Enzyme promiscuity describes the capability of an enzyme to catalyze various reactions, i.e., besides the catalysis of the main reaction, for which it has been evolved during evolution, also various other reactions are catalyzed (Khersonsky and Tawfik, 2010; Copley, 2014). Enzyme

promiscuity might be achieved by several mechanisms, such as a conformational change in the active site, binding variable substrates, or different co-factors (Khersonsky and Tawfik, 2010; Copley, 2014).

In conclusion, it seems to be very likely that the modification of xenobiotics taken up by the plants is mainly due to reactions accidentally catalyzed by promiscuous enzymes involved in the genuine secondary metabolism of acceptor plants rather than by processes related to "deliberate detoxification" processes as outlined by the "Green Liver Concept". Nonetheless, although the principle is different, the outcome is very similar: when xenobiotics enter the plants, they could be modified and thereby detoxified differentially according to the plant species (Schulz and Wieland, 1999; Shajib et al., 2012). In this context, especially with respect to a rapidly changing environment, promiscuity of enzymes represents an important factor of further adaptations (Schwab, 2003).

5.5 Ecological significance

The results shown and discussed above, states that besides alkaloids, also phenolic substances are taken up and modified within the acceptor plants. Hitherto, most of the research interest was concentrated on the natural compounds that have a certain effect, i.e., allelochemicals. Only the compounds leached or exuded from plants, which reveal an ecological function, e.g., inhibition of the germination or growth of other plants grown in the vicinity, are taken into consideration (Einhellig, 1995).

It is worth mentioning that the uptake of the natural compounds by plants is a general phenomenon, regardless of their biological function. Also, the ability to biotransform these compounds differs between plant species (Hijazin et al., 2019).

Up to now, any ecological significance of the uptake and modification of a certain compound could not be predicted. Indeed, the corresponding

concentrations of umbelliferone and its derivatives in the acceptor plants are in the same range as those in putative donor plants (Petruľova-Poracka et al., 2013). However, if we assume that the actual concentrations in the soil due to putative leaching processes might be far lower than the umbelliferone concentration used in the previous experiments, no solid statements on the content in the acceptor plants in the field could be made. Accordingly, up to now, any implication on putative ecological significances could not be predicted (Hijazin et al., 2019).

It is well established that allelochemicals like juglone, biochanin A or BOA, are taken up by plants (Schulz and Wieland, 1999; Shajib et al., 2012). However, a limited number of studies showed that these allelochemicals are taken up and modified in the acceptor plants, and these modifications changed their effect (Schulz and Wieland, 1999; Shajib et al., 2012). Also as stated in this study, umbelliferone is modified differentially within the various plant species (Hijazin et al., 2019). As the inhibitory effect of juglone strongly varies between different plant species (e.g., Rietveld, 1982; Williams and Hoagland, 1982; Kocace Aliskan and Terzi, 2001). Thus, it could be assumed that the differential modification of juglone within various plant species, strongly affects its inhibitory effects on the related species: i.e., juglone strongly inhibits certain metabolic events in certain plants, whereas in other species no inhibitory effect could be detected (Hijazin et al., 2019). Accordingly, further promising experimental approaches are required. Nonetheless, we have to take into consideration that such investigations might be complicated since putative derivatives of juglone- in contrast to umbelliferone- are unknown and hard to be identified (Hijazin et al., 2019).

However, an alternative approach could be employed to reveal the relation between the differential modification and the impact of an allelochemical, by using

certain inhibitors for these modifications. Since the derivatization of umbelliferone could be inhibited by naproxen -a typical P450 enzyme inhibitor- also the modifications of juglone could be inhibited by naproxen. In this context, the simultaneous application of naproxen and juglone could inhibit its modifications and therefore restore its inhibitory effects on the related plant species (Hijazin et al., 2019).

Summary

According to the recently discovered phenomenon of "*Horizontal Natural Product Transfer*", alkaloids, which are leached out from rotting plant parts or are exuded from living plants into the soil, are taken up by plants growing in the vicinity. The question arose, if other natural compounds are imported in the same manner. In this thesis, this subject was investigated fundamentally focusing on phenolic natural compounds. Based on its high stability against oxidation and its intensive fluorescence, umbelliferone was chosen as an eminently suitable model compound. Its uptake and derivatization were investigated by employing seedlings of various plant species grown in an optimized hydroponic system.

The seedlings of all plant species tested, took up umbelliferone by their roots and translocated it into the leaves. However, depending on the plant species, the metabolic fate of umbelliferone was quite different. In seedlings of pea (*Pisum sativum* L.), flax (*Linum usitatissimum* L.), and radish (*Raphanus sativus* L.), the imported umbelliferone was just accumulated in the leaves to a high extent. In contrast, in the seedlings of barley (*Hordeum vulgare* L.) and garden cress (*Lepidium sativum* L.), umbelliferone was modified by hydroxylation, methylation, and glucosylation. In barley, these modifications yield in the production of high amounts of scopoletin (6-methoxy-7-hydroxycoumarin, representing a "*6-Methoxyumbelliferone*"), whereas in garden cress esculin (6,7-Dihydroxycoumarin 6-glucoside) was generated, which corresponds to a "*glucosyl-hydroxy-umbelliferone*". In principle, there are several options for the order of the various modifications. Accordingly, different putative intermediates, i.e., skimmin or esculetin could be involved. Yet, it was found that esculetin (6,7-dihydroxycoumarin, representing "*hydroxy-umbelliferone*") is generated as an intermediate in both plant species. Obviously, in both plants, umbelliferone is

firstly hydroxylated to yield esculetin. Then, in barley, it is methylated to scopoletin, whereas in garden cress it is glucosylated to yield esculin.

For further elucidation of the conversion of umbelliferone to its derivatives, esculetin - the putative intermediate - was also applied to the tested seedlings. In contrast to umbelliferone, esculetin was oxidized very rapidly in the growth medium, resulting in a black melanin-like layer around the roots. This circumstance seems to be the reason, why the coumarin is not taken up by the roots of pea and flax seedlings. In contrast, the seedlings of garden cress, barley, and radish did. However, in these seedlings, the imported esculetin was derivatized efficiently. Whereas in barley esculetin was methylated to scopoletin, in *Lepidium* and *Raphanus* esculetin was glucosylated to yield esculin.

The production of these derivatives is fully compatible with the proposed reaction pathways, expounding esculetin as an intermediate of the umbelliferone modification in barley and garden cress. Moreover, although in radish umbelliferone is not derivatized, esculetin is glucosylated. This implies that the enzymes capable of hydroxylating umbelliferone are missing in radish.

Due to its physicochemical properties, esculetin has to be taken up by all acceptor plants. Obviously, the blackening reactions prevent the uptake of esculetin, either by decreasing its availability due to the oxidation or by hindering its diffusion through the black melanin-like layer around the roots. Unfortunately, the various approaches to provoke an esculetin uptake in flax and pea seedlings failed, e.g., by adding ascorbic acid as an antioxidant to reduce esculetin oxidation, or by an ongoing supply by adding esculin, from which esculetin is liberated by the action of apoplastic β-glucosidases.

The finding that the imported coumarins are derivatized raised the question, where such modifications take place. Accordingly, various approaches have been performed. Firstly, it was investigated whether the genuine imported coumarins

or their derivatives are translocated from the roots into the shoots. A corresponding translocation should be performed via xylem. Accordingly, guttation droplets, which represent xylem sap, were analyzed. It turned out, that in barley as well as in garden cress, umbelliferone and scopoletin are present in the guttation droplets. However, the glucoside esculin generated in garden cress was not, pointing to the fact that the glucosylation in garden cress occurs in the leaves. In contrast, the presence of scopoletin did not affirm the site of its production, since this coumarin is passively diffusible across biomembranes. Accordingly, scopoletin, which might be generated in the leaves, will also be present in the guttation droplets due to its distinctive ability to cross biomembranes. This high diffusibility was vividly demonstrated by the presence of umbelliferone in tissue paper wrapped around shoots of barley control plants.

Thus, another approach was performed by analyzing the capability of discrete organs to modify the imported umbelliferone. Accordingly, excised leaves and roots were incubated with umbelliferone. In garden cress, umbelliferone was hydroxylated and glucosylated in the roots as well as in the leaves. However, due to the absence of this glucoside in the xylem, it can be deduced that the esculin accumulated in the leaves results from modification processes in the leaves.

In barley, no scopoletin was generated in the roots, whereas high amounts of this derivative were produced in the leaves, verifying that the scopoletin found in the seedlings growing in the umbelliferone-containing medium is produced in the leaves.

Astonishingly, in the excised barley leaves, an unusual pattern of umbelliferone derivatives was detected. In addition to scopoletin, huge amounts of glucosides, i.e., esculin and scopolin are accumulated, too. Obviously, the huge amounts of scopoletin and its intermediate esculetin are glucosylated in the excised barley leaves when incubated with umbelliferone. This, however, outlines that enzymes

capable to glucosylate these coumarins must be active. Unfortunately, up to now, no clues are available, why these glucosyltransferases are activated in the excised leaves, whereas they are not in the entire barley seedlings.

The conversion of umbelliferone to esculetin requires a hydroxylation step, which frequently is catalyzed by cytochrome P450 enzymes. Corresponding enzymes are well known to be involved in the detoxification of xenobiotics. A suitable approach to elucidate the involvement of cytochrome P450 hydroxylases is based on their inhibition by specific inhibitors, such as naproxen. Accordingly, umbelliferone was applied together with naproxen. As expected, in barley the conversion of umbelliferone to scopoletin as well as the generation of esculin in garden cress was strongly reduced by the presence of naproxen, verifying that in both plants, hydroxylation of umbelliferone is catalyzed by cytochrome P450 enzymes.

The data elaborated in this thesis for the first time demonstrate that – in addition to alkaloids – also phenolic compounds are taken up by various acceptor plants and subsequently are modified. However, the metabolic fate strongly depends on the plant species. Whereas in some plants the imported coumarin is just accumulated in their leaves, it is differentially modified in others. Altogether, these findings imply that the horizontal natural product transfer represents a more general phenomenon.

In addition to the relevance for plant ecology, the coherences elaborated in this thesis also might have a great impact on our general understanding of detoxification processes of xenobiotics. Up to now, our perception of these processes is characterized by the so-called "*green liver concept*". According to this theory, substances are detoxified "*deliberately*" in the acceptor plants. But, the results on the species-specific and differential modifications of imported coumarins point to the assumption that the observed modifications are due to the incidental presence of enzymes capable to catalyze them. In other words, these modifications seem to occur accidentally rather than target-oriented.

Zusammenfassung

Gemäß dem kürzlich entdeckten Phänomen des "*Horizontalen Naturstofftransfers*" werden Alkaloide, die aus verrottenden Pflanzenteilen ausgelaugt oder von lebenden Pflanzen in den Boden abgegeben werden, von in der Nähe wachsenden Pflanzen aufgenommen. Es stellte sich die Frage, ob auch andere Naturstoffe in gleicher Weise in Pflanzen importiert werden. In dieser Arbeit wurde diese Thematik intensiv untersucht; dabei lag der Schwerpunkt auf der Aufnahme von Cumarinen, die exemplarisch für weitere phenolische Naturstoffen eingesetzt wurden. Für die meisten Untersuchungen wurde Umbelliferon verwendet, da dieses Cumarin eine relativ hohe Widerstandsfähigkeit gegenüber Oxidationen aufweist und aufgrund seiner intensiven Fluoreszenz sehr gut detektiert werden kann. Zur Untersuchung der Aufnahme und Derivatisierung von Umbelliferon wurde ein geeignetes hydroponisches System entwickelt und optimiert. Hiermit konnten Sämlinge unterschiedlicher Pflanzenarten kultiviert und unterschiedlichen Cumarin-Konzentrationen ausgesetzt werden.

Die Sämlinge aller getesteten Pflanzenarten haben Umbelliferon über ihre Wurzeln aufgenommen und in die Blätter verlagert. Je nach Pflanzenart war das weitere Schicksal von Umbelliferon jedoch sehr unterschiedlich. In den Sämlingen von Erbsen (*Pisum sativum* L.), Flachs (*Linum usitatissimum* L.) und Rettich (*Raphanus sativus* L.) wurde das importierte Umbelliferon lediglich in hohem Maße in den Blättern akkumuliert. Im Gegensatz dazu wurde Umbelliferon in den Sämlingen von Gerste (*Hordeum vulgare* L.) und Gartenkresse (*Lepidium sativum* L.) durch Hydroxylierungen, Methylierungen und Glucosylierungen modifiziert. In Gerste resultierte aus diesen Modifikationen die Bildung großer Mengen an Scopoletin (6-Methoxy-7-hydroxycumarin, das formal auch als ein "*6-Methoxyumbelliferon*" angesehen werden kann), während in Gartenkresse Esculin (6,7-di-Hydroxycumarin-6-glucosid) gebildet wurde, das formal auch als "*6-*

Glucoxy-Umbelliferon" bezeichnet werden kann. Grundsätzlich gibt es mehrere Möglichkeiten für die Reihenfolge der verschiedenen Modifikationen. Dementsprechend könnten verschiedene mutmaßliche Zwischenprodukte, wie Skimmin oder Esculetin, im Zuge der Modifikationen entstehen. Die Ergebnisse haben gezeigt, dass in beiden Pflanzenarten Esculetin (6,7-Dihydroxycumarin) als Zwischenprodukt generiert wird. Offensichtlich wird in beiden Pflanzen das importierte Umbelliferon zunächst hydroxyliert. Das dabei entstehende Esculetin wird in Gerste dann zu Scopoletin methyliert, während es in der Gartenkresse zu Esculin glucosyliert wird.

Zur weiteren Aufklärung der Modifizierung von Umbelliferon wurde auch Esculetin - das mutmaßliche Zwischenprodukt – den Sämlinge appliziert. Im Gegensatz zum Umbelliferon wurde das Esculetin jedoch im Medium sehr schnell oxidiert. Dabei bildet sich eine schwarze, melaninähnliche Schicht um die Wurzeln der Keimlinge. Dies scheint der Grund dafür zu sein, dass Esculin nicht von den Wurzeln der Lein- und Erbsensämlinge aufgenommen wird. Im Gegensatz dazu haben die Sämlinge der anderen drei getesteten Arten (Gartenkresse, Gerste und Rettich) das Cumarin aufgenommen. Allerdings wurde das importierte Esculetin sehr effizient derivatisiert. Dabei wurde es in den Gerstesämlingen zu Scopoletin methoxyliert, während es in den Sämlingen von *Lepidium* und *Raphanus* zu Esculin glucosyliert wurde. Diese Ergebnisse bestätigen die postulierten Reaktionswege mit Esculetin als Zwischenprodukt. Auch in Radieschen wird – im Gegensatz zur einfachen Akkumulation des importierten Umbelliferons - das aufgenommene Esculetin zu Esculin glucosyliert. Dies legt nahe, dass die Enzyme, die in der Lage sind Umbelliferon zu hydroxylieren, in Radieschen nicht vorhanden sind.

Aufgrund seiner physiko-chemischen Eigenschaften muss Esculetin prinzipiell von allen Akzeptorpflanzen aufgenommen werden. Offensichtlich verhindern die Bräunungsreaktionen die Aufnahme von Esculetin; entweder weil sie seine

Verfügbarkeit aufgrund der Oxidation stark vermindern, oder weil seine Diffusion durch die schwarze, melaninähnliche Schicht um die Wurzeln stark behindert wird. Leider waren die verschiedenen Ansätze, eine Aufnahme von Esculetin in Rettich- und Erbsensämlingen zu forcieren, erfolglos: weder die Zugabe von Ascorbinsäure (als Antioxidans zur Unterdrückung der Oxidation von Esculetin) noch die Applikation von Esculin (um eine langfristige, kontinuierliche Versorgung mit Esculetin aufgrund seiner Freisetzung durch die Aktivität apoplastischer ß-Glucosidasen zu gewährleisten) führte zu einer Aufnahme von Esculetin in die Rettich- und Erbsensämlinge.

Der Befund, dass die importierten Cumarine in einigen Sämlingen derivatisiert werden, warf die Frage auf, wo diese Modifikationen stattfinden. Um diese Frage zu klären, wurden verschiedene Ansätze durchgeführt. Zunächst wurde untersucht, ob die importierten Cumarine selbst oder deren Derivate von den Wurzeln in die Blätter verlagert werden. Eine derartige Verlagerung sollte über das Xylem erfolgen. Dementsprechend wurden Guttationstropfen, die dem Xylemsaft entsprechen, analysiert. Es stellte sich heraus, dass sowohl in Gerste als auch in Gartenkresse Umbelliferon und Scopoletin in der Guttationsflüssigkeit vorhanden sind, aber nicht das Glucosid Esculin. Dies legt nahe, dass in der Gartenkresse die Glucosylierung der Cumarine in den Blättern stattfindet. Im Gegensatz zu dieser Schlussfolgerung kann das Vorhandensein von Scopoletin in der Guttationsflüssigkeit nicht als eindeutiger Beweis dafür gewertet werden, dass eine Verlagerung von Scopoletin aus den Wurzeln in die Blätter stattfindet. Aufgrund seiner Eigenschaft passiv über Biomembranen zu diffundieren würde auch Scopoletin, das in den Blättern gebildet wird, in den Guttationstropfen vorhanden sein. Eine entsprechend ausgeprägte Diffusion wurde anschaulich durch das Vorhandensein von Umbelliferon in den Papiersteifen, die um die Triebe der Gerstenkontrollpflanzen gewickelt war, belegt.

Alternativ wurde ein anderer Ansatz durchgeführt, bei dem die Fähigkeit der einzelner Organe analysiert wurde, das importierte Umbelliferon zu modifizieren. Dementsprechend wurden ausgeschnittene Blätter und Wurzeln mit Umbelliferon inkubiert. Dabei zeigte sich, dass in Gartenkresse das Umbelliferon sowohl in den Wurzeln als auch in den Blättern hydroxyliert und glucosyliert wurde und als Esculin akkumuliert. Allerdings kann aufgrund des Fehlens dieses Glucosids im Xylem geschlussfolgert werden, dass das in den Blättern akkumulierte Esculin in den Blättern gebildet wird.

In den Wurzeln der Gerste wurde so gut wie kein Scopoletin gebildet, während große Mengen dieses Derivats in den Blättern produziert wurden. Dies zeigt, dass das Scopoletin, das in den Blättern der Sämlinge nach Umbelliferon-Aufnahme akkumuliert wird, in den Blättern gebildet wird. Im Gegensatz zu den Versuchen mit intakten Sämlingen resultierte bei der Inkubation abgeschnittener Gerstenblätter allerdings ein sehr komplexes Spektrum von Umbelliferon-Derivaten. Neben Scopoletin zeigten sich große Mengen der Glucoside Esculin und Scopolin. Offensichtlich werden in den abgeschnittenen Gerstenblättern großen Mengen an Scopoletin und dem vermeintlichem Zwischenprodukt Esculetin glucosyliert. Das bedeutet, dass die Enzyme, die Cumarine glucosylieren können, tatsächlich aktiv sind. Leider gibt es bisher keine Hinweise, warum diese Glucosyltransferasen in den ausgeschnittenen Blättern aktiv und in den intakten Gerstensämlingen inaktiv sind.

Die Umwandlung von Umbelliferon in Esculetin entspricht einer Hydroxylierung - eine Reaktion, die häufig durch Cytochrom P450-Enzyme katalysiert wird. Es ist bekannt, dass entsprechende Enzyme auch an der Entgiftung von Xenobiotika beteiligt sind. Ein geeigneter Ansatz zur Aufklärung der Beteiligung von Cytochrom P450-Hydroxylasen basiert auf ihrer Hemmung durch spezifische Inhibitoren wie Naproxen. Dementsprechend wurde Umbelliferon zusammen mit

Naproxen den Sämlinge appliziert. Wie erwartet wurde bei Gerste die Umwandlung von Umbelliferon zu Scopoletin sowie in Gartenkresse die Bildung zu Esculin durch die Anwesenheit von Naproxen stark reduziert. Dies legt nahe, dass in beiden Pflanzen die Hydroxylierung von Umbelliferon zu Esculetin durch Cytochrom P450-Enzyme katalysiert wird.

Die in dieser Studie erarbeiteten Daten zeigen erstmals, dass neben Alkaloiden auch phenolische Naturstoffe von den Wurzeln der Pflanzen aufgenommen werden. In einigen Akzeptorpflanzen werden die Cumarine anschließend modifiziert, d.h., das metabole Schicksal der importierten Substanzen hängt stark von der Pflanzenart ab. Während in einigen Pflanzen das importierte Cumarin lediglich in den Blättern akkumuliert wird, wird es in anderen unterschiedlich modifiziert. Insgesamt deuten diese Ergebnisse darauf hin, dass der horizontale Naturstofftransfer ein weit verbreitetes, allgemeines Phänomen darstellt.

Neben der Relevanz für die Pflanzenökologie könnten die in dieser Studie erarbeiteten Erkenntnisse auch einen großen Einfluss auf unser allgemeines Verständnis der Entgiftungsprozesse von Xenobiotica haben. Bisher ist unsere Wahrnehmung dieser Prozesse durch das sogenannte "*Green liver concept*" gekennzeichnet. Nach dieser Theorie werden Substanzen in den Akzeptorpflanzen "*gezielt*" entgiftet. Die Ergebnisse zu den artspezifischen, unterschiedlichen Modifikationen importierter Cumarine deuten allerdings darauf hin, dass die beobachteten Modifikationen lediglich auf ein zufälliges Vorhandensein von Enzymen zurückzuführen sind, die in der Lage sind, entsprechende Reaktionen zu katalysieren. Mit anderen Worten, diese Modifikationen scheinen eher zufällig als zielorientiert zu sein.

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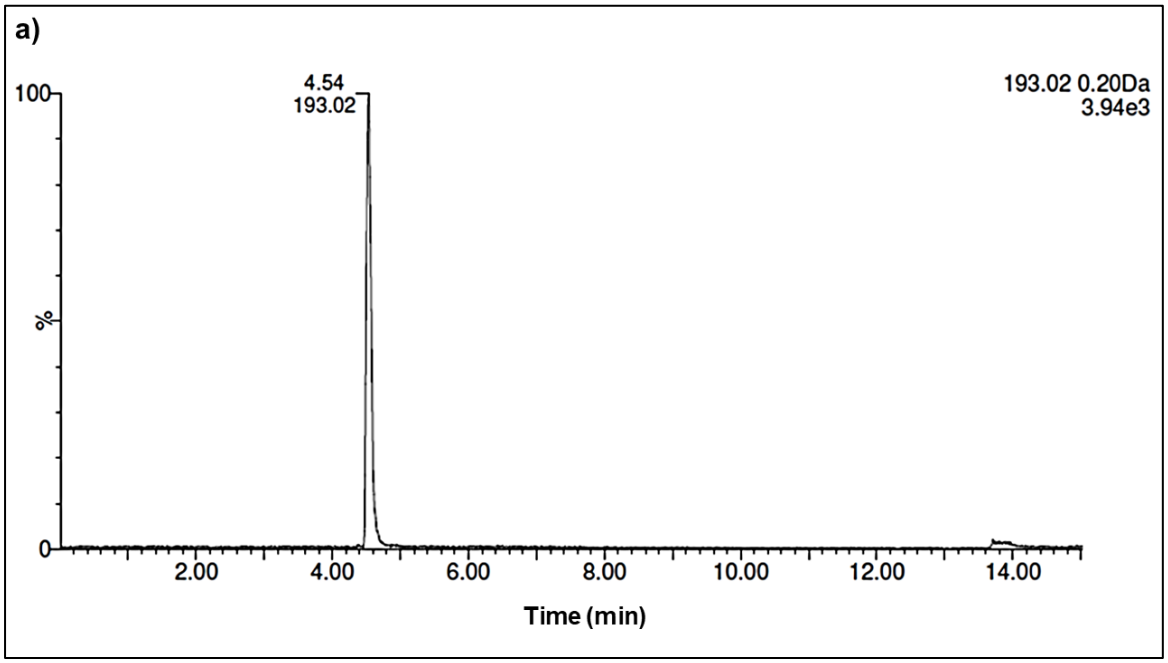
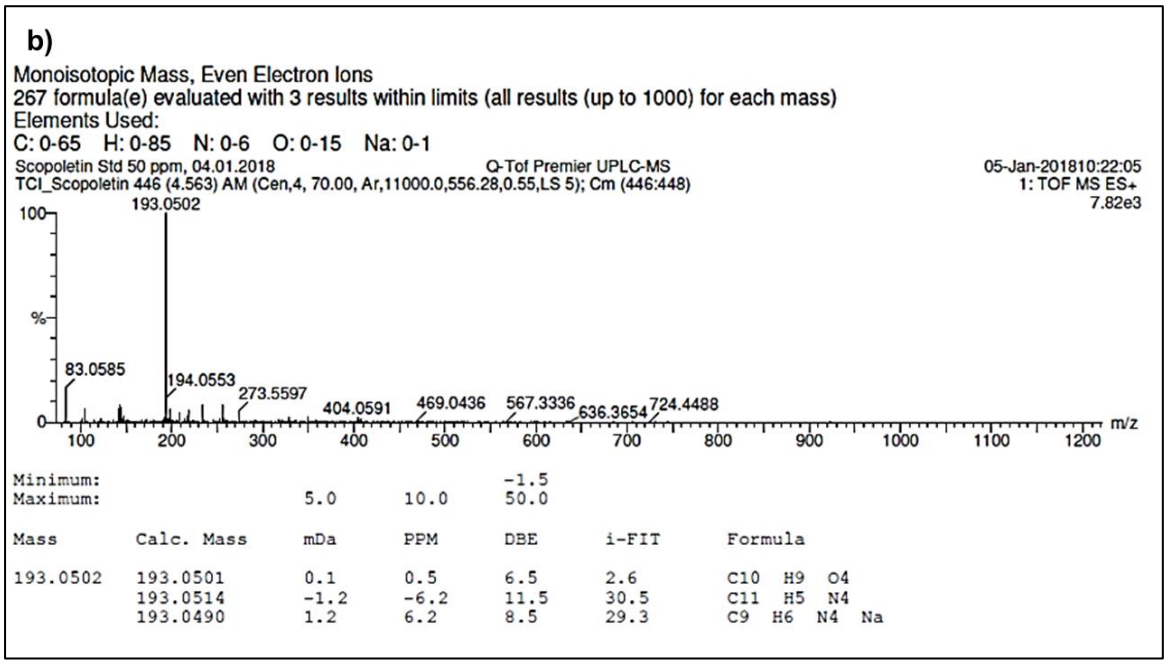
Appendix

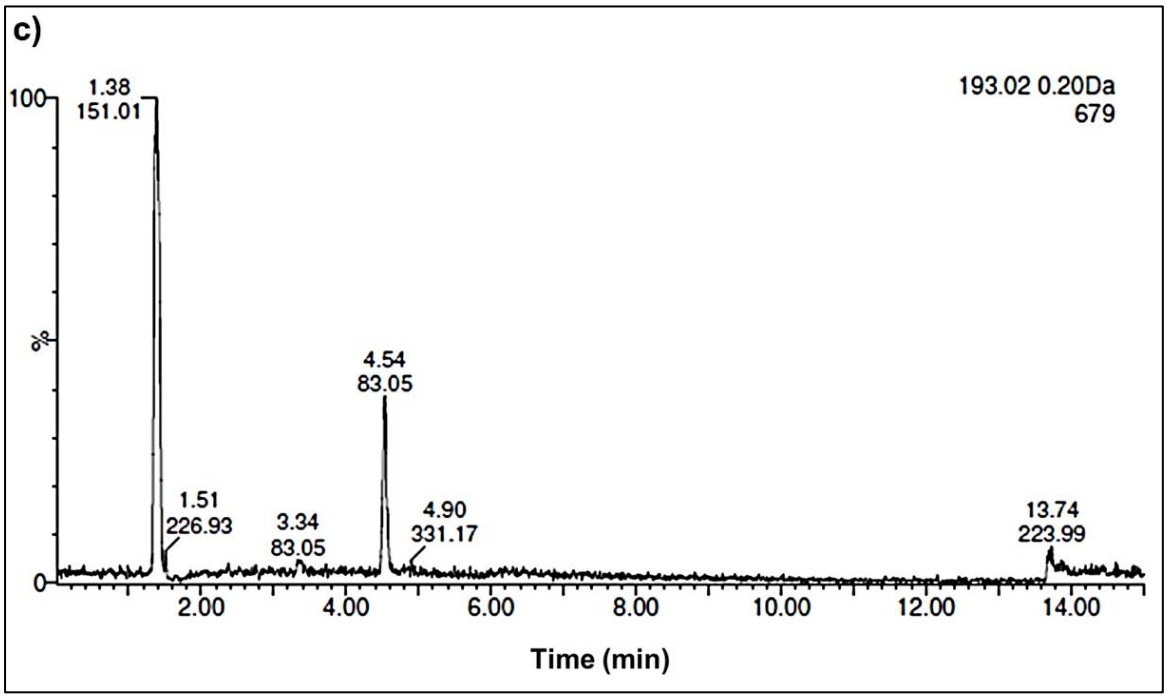
Formation of melanin-like layer on the barley seedling roots due to the oxidation of the applied esculetin or esculin in the medium

Figure A-1: Blackening of the barley seedling roots due to the oxidation of the applied esculetin or esculin in the medium (left) comparing to control barley seedling roots (right).

LC-MS analysis of scopoletin







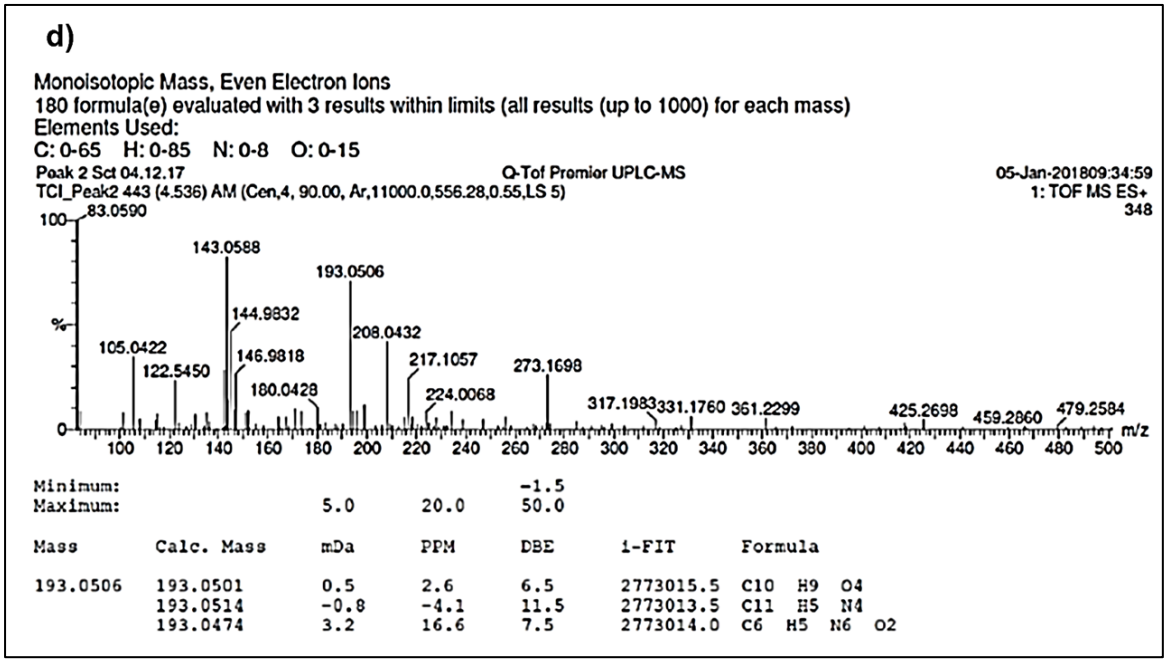
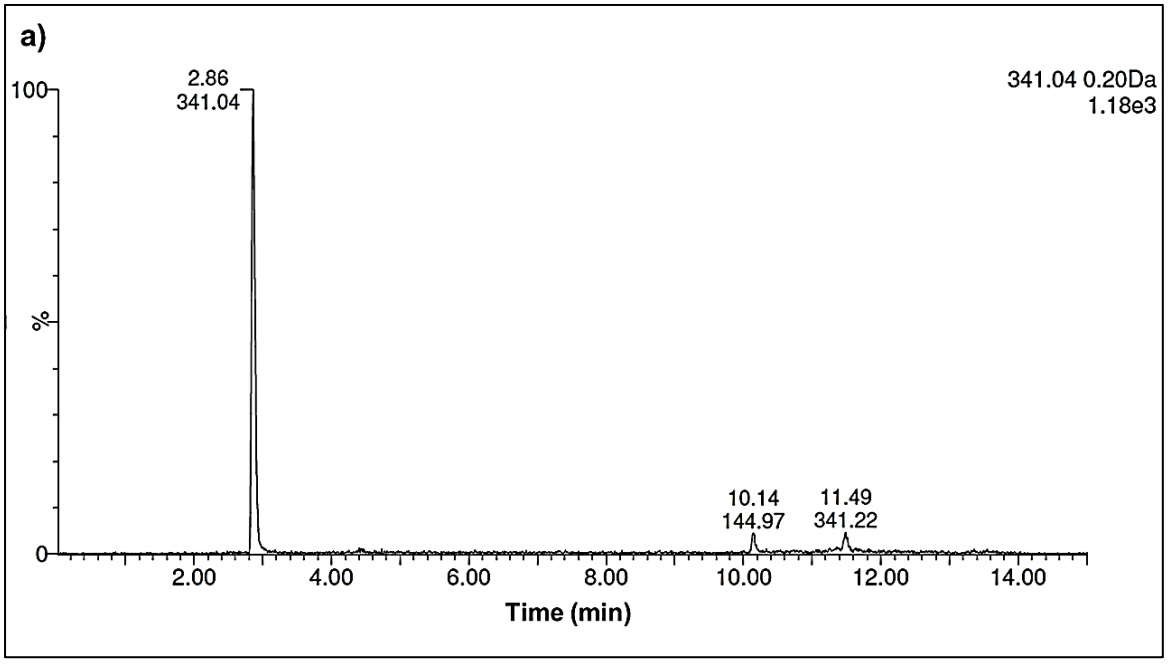
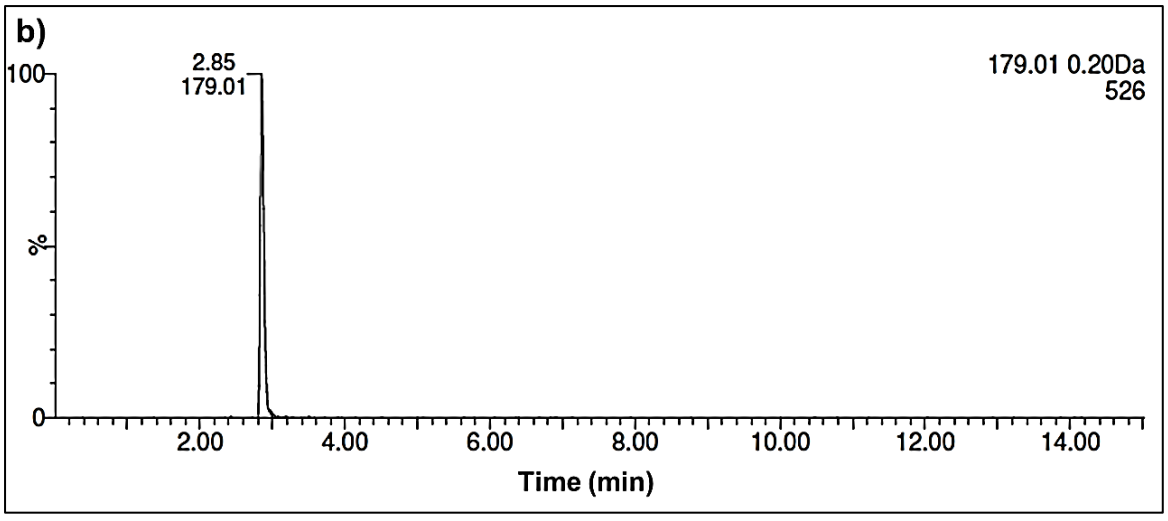


Figure A-2: LC-MS of scopoletin. a) Ion chromatogram (M+H+) for scopoletin (authentic standard); b) high resolution ESI-MS for scopoletin (authentic standard); c) Ion chromatogram (M+H+) for scopoletin (isolated from barley); d) high resolution ESI-MS for scopoletin (isolated from barley).

LC-MS analysis of esculin

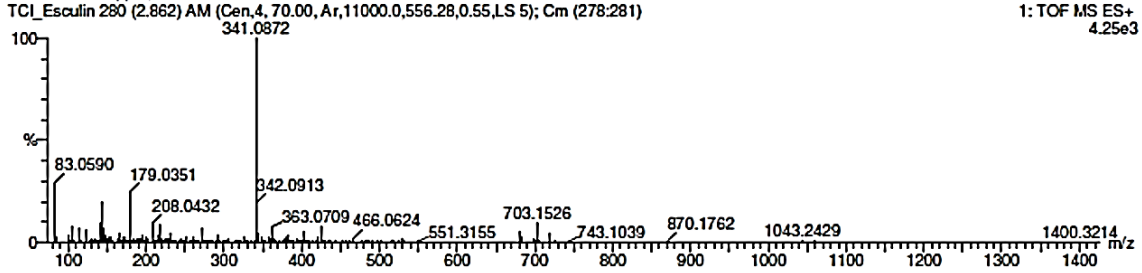




c)

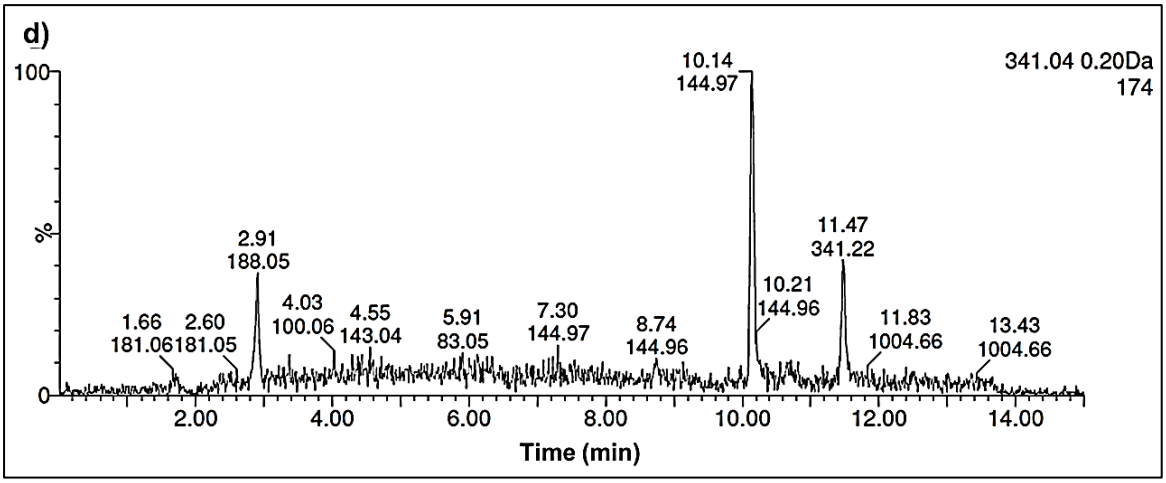
Monoisotopic Mass, Even Electron Ions
706 formula(e) evaluated with 7 results within limits (all results (up to 1000) for each mass)
Elements Used:
C: 0-65 H: 0-85 N: 0-6 O: 0-15 Na: 0-1

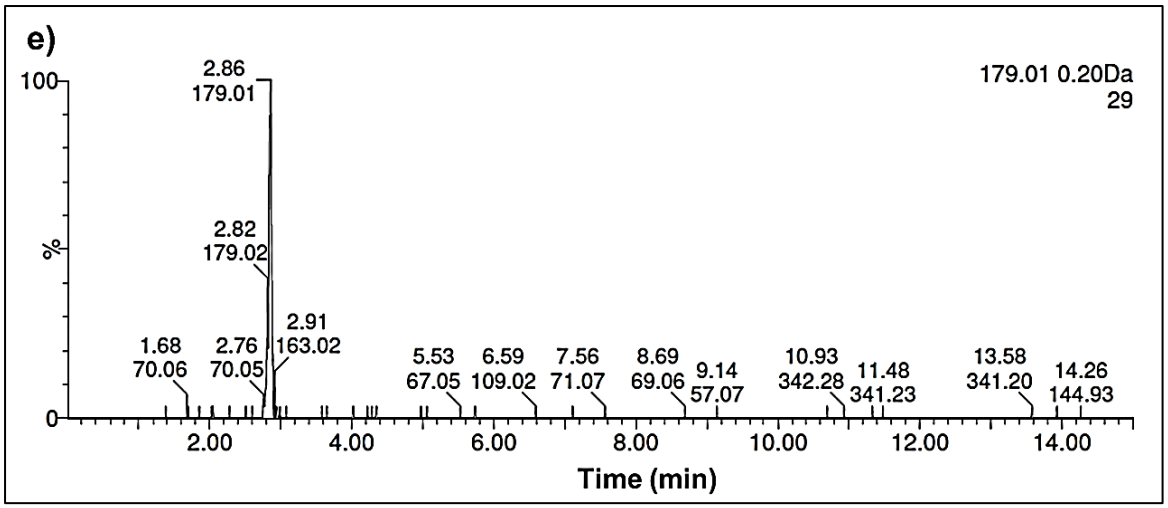
Esculin Std 50 ppm, 04.01.2017 Q-Tof Premier UPLC-MS 05-Jan-201810:37:45
TCI_Esculin 280 (2.862) AM (Cen,4, 70.00, Ar,11000.0,556.28,0.55,LS 5); Cm (278:281) 1: TOF MS ES+
4.25e3



Minimum:				-1.5		
Maximum:		5.0	10.0	50.0		
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Formula
341.0872	341.0873	-0.1	-0.3	7.5	6.5	C15 H17 O9
	341.0862	1.0	2.9	9.5	15.2	C14 H14 N4 O5 Na
	341.0886	-1.4	-4.1	12.5	7.1	C16 H13 N4 O5

d)





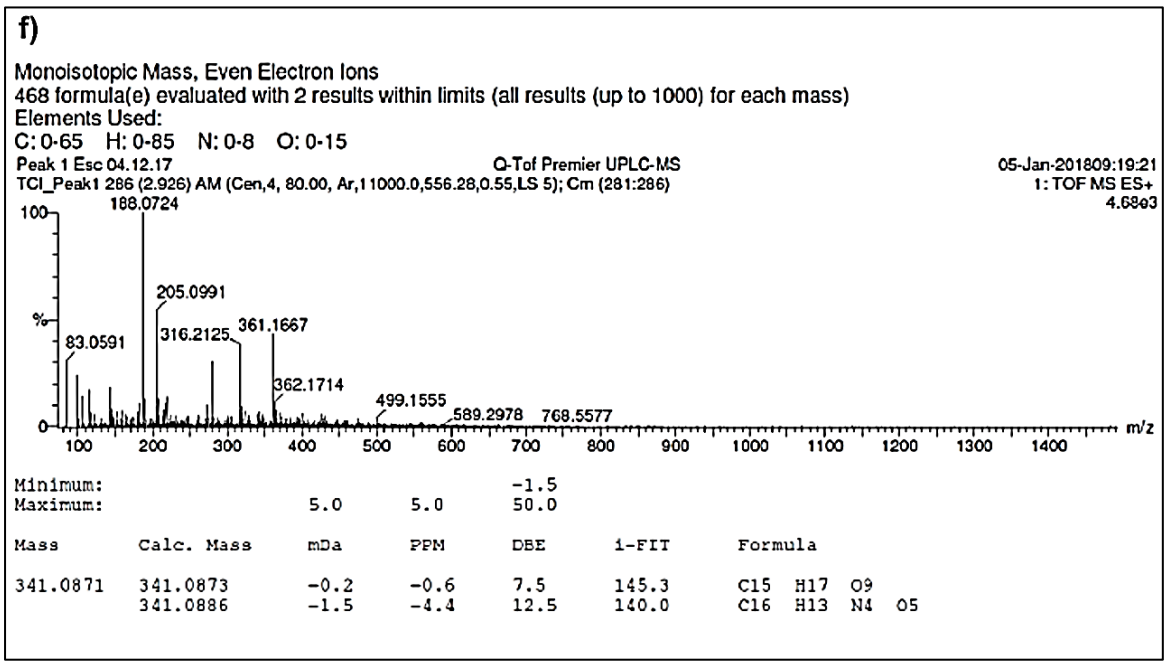


Figure A-3: LC-MS of esculin: a) Ion chromatogram (M+H+) for esculin (authentic standard); b) MS/MS Ion chromatogram (main fragment m/z = 179) for esculin (authentic standard); c) high resolution ESI-MS for esculin (authentic standard); d) Ion chromatogram (M+H+) for esculin (isolated from garden cress); e) MS/MS Ion chromatogram (main fragment m/z = 179) for esculin (isolated from garden cress); f) high resolution ESI-MS for esculin (isolated from garden cress).

Depletion of esculetin from the medium due to its oxidation

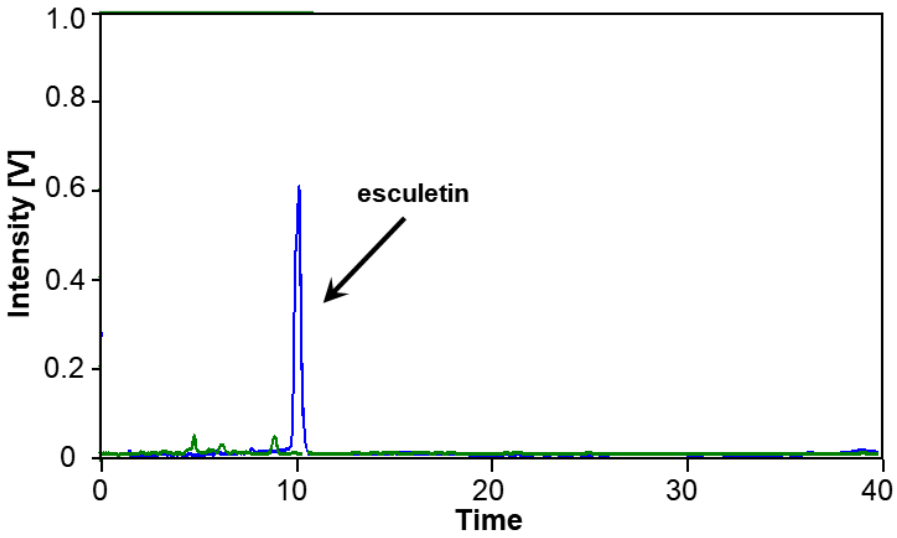


Figure A-4: HPLC analysis of esculetin-containing medium in which the barley seedlings were grown. The corresponding HPLC chromatogram of the medium on the 2nd day of incubation is shown in blue, this of the 5th day is shown in green.

Hydrolyzing of esculin by glucosidases

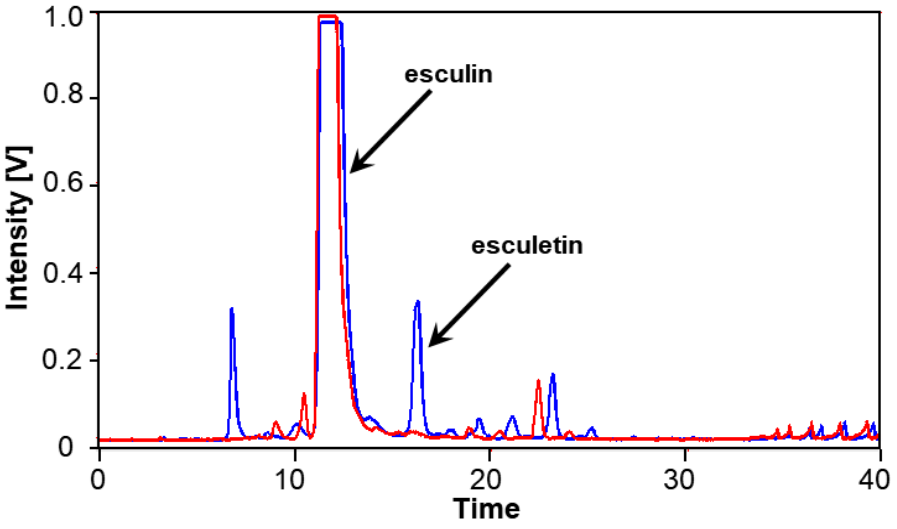


Figure A-5: Liberation of esculetin from its glucoside (esculin) in the media. The corresponding HPLC chromatogram of the extract from plants treated with only esculin is displayed in blue, this of the plants treated with esculin and glucosidase inhibitors is given in red.

Diminishing of umbelliferone uptake due to the formation of melanin-like layer on the seedling roots.

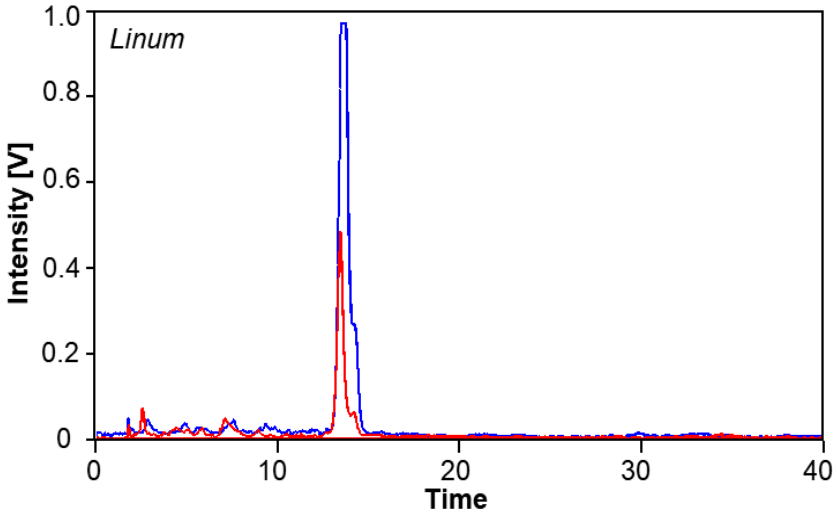


Figure A-6: Esculetin oxidation decreases the ability of flax seedlings (*Linum usitatissimum*) to import coumarins by damaging their roots through the formation of melanin-like layer. The corresponding HPLC chromatogram of the extract from the plants treated with only umbelliferone is displayed in blue, this of the plants treated with esculetin and umbelliferone is given in red.

Ascorbic acid didn't enhance esculetin uptake by flax seedlings

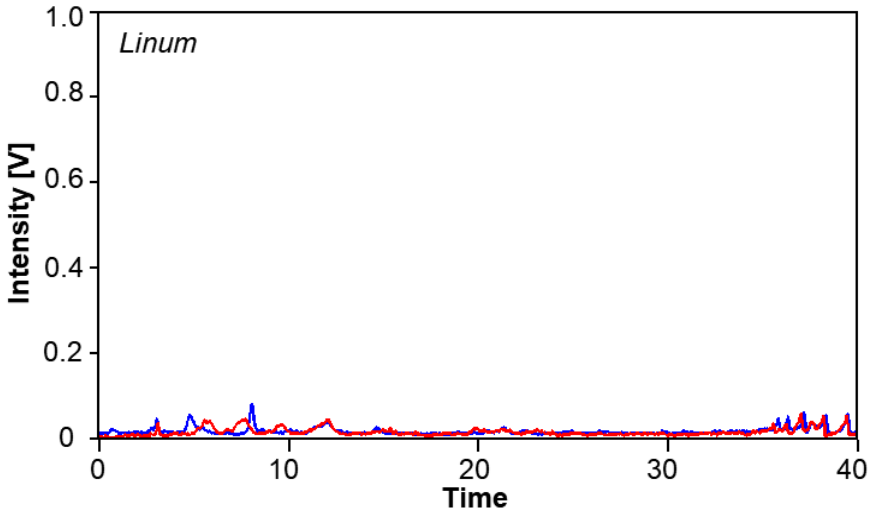


Figure A-7: Effect of the ascorbic acid on the uptake of esculetin by flax seedlings (*Linum usitatissimum*). The corresponding HPLC chromatogram of the extract from plants treated with only esculetin is displayed in blue, this of plants treated with esculetin and ascorbic acid is given in red.

Curriculum Vitae

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- 2000-2004, Bachelor's degree in Biotechnology and Genetic Engineering, Jordan University of Science and Technology, Jordan, (90.9).
- 2011-2014, Master's degree in biology, Mutah University, Jordan (94.75).

Experiences:

- From 9/5/2004 to 9/5/2005:

  Working as lab technician in Princess Haya Biotechnology Center at Jordan University of Science and Technology

- From 23/11/2005- 31/12/2016:

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Language:

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- English

Publications:

1. **Hijazin T**, Radwan A, Lewerenz L, Abouzeid S, Dräger G, Selmar D. (2020) The uptake of alkaloids by plants from the soil is determined by rhizosphere pH. ***Rhizosphere*** 15: 100234.

2. Lewerenz L, **Hijazin T**, Abouzeid S, Hänsch R, Selmar D. (2020) Pilot study on the uptake and modification of harmaline in acceptor plants: An innovative approach to visualize the interspecific transfer of natural products. ***Phytochemistry*** **174**:102-107.

3. Selmar, D., Abouzeid, S., Radwan, A., **Hijazin, T**., Yahyazadeh, M., Lewerenz, L., Nowak, M., Kleinwächter, M. (2020) Horizontal Natural Product Transfer - A Novel Attribution in Allelopathy. In. Mérillon, J-M., Ramawat, K.G. (Eds.): ***Reference Series in Phytochemistry***. Co-Evolution of Secondary Metabolites: 429 – 439.

4. **Hijazin, T**., Radwana,A., Abouzeid,S., Dräger,G., Selmar, D. (2019) Uptake and modification of umbelliferone by various seedlings. ***Phytochemistry*** 157: 194–199.

5. Selmar, D., Radwan, A., **Hijazin, T**., Abouzeid, S., Yahyazadeh, M, Lewerenz, L., Kleinwächter, M., Nowak, M. (2019) Perspective Horizontal Natural Product Transfer: Intriguing Insights into a Newly Discovered Phenomenon. ***Journal of Agricultural and Food Chemistry*** **67**: 8740-8745**.**

6. Abouzeid, S., **Hijazin, T**., Lewerenz, L., Hänsch, R., Selmar, D. (2019) The genuine localization of indole alkaloids in Vinca minor and *Catharanthus roseus*. ***Phytochemistry*** **168**:102-107**.**

Workshops & Conferences

- DAAD – EXCEED Regional Workshop on "Wastewater Treatment and Reuse" Konya -Turkey at 03 – 06 June 2013.

- Humboldt Kolleg "Building International Networks for Enhancement of Research in Jordan " Princess Sumaya University Amman, April 3-5, 2014.

- Oral presentation: Hijazin, T., "Horizontal Natural product Transfer: Uptake and modification of umbelliferone". Workshop of the "German. Botanical Society – Section Natural Products" Burg Warberg, 2018, October 1st – 3rd.

- Oral presentation: Hijazin, T., Selmar, D. "Horizontal Natural product Transfer: Uptake and modification of coumarins". Congress of the German Botanical Society, Rostock, 2019, September 16th – 19th.